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The Role of MMP-2 and MMP-14 in Cell Migration and Invasion

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ACKNOWLEDGEMENTS

ABBREVIATIONS

MMP   Matrix metalloproteinase

DMSO   Dimethyl sulfoxide

DTT   Dithiothrietol

ECL   Enhanced chemilluminiscence

E.coli   Escherichia coli

EDTA   Ethylene diamine tetraacetic acid

FBS   Fetal bovine serum

HRP   Horseradish peroxidase

KdA   Kilo Dalton

PAGE   Polyacrylamide gel electrophoresis

PBS   Phosphate buffered saline

PCR   Polymerase chain reaction

SDS   Sodium dodecyl sulphate

shRNa   Short hairpin RNA

siRNA   Short interfering RNA

DMEM-F12   Dulbecco's Modified Eagle's Medium

MMP-2ic   Intracellular MMP-2

gDNA   Genomic DNA

BSA   Bovine serum albumin

TIMP   Tissue inhibitor of matrix metalloproteinases

DQ-gelatin   Dye quenched gelatin

SFM   Serum-free medium

SUMMARY

Cancer is becoming a global epidemic in recent times and can affect different organs in the body and even more worrying is that they do not have a common cause. Several factors have been implicated in the onset and spread of cancers, one of which are the metalloproteinases (MMPs). The MMPs utilize the same strategy they employ in normal physiological processes in pathology, thus creating a dilemma in the development of cancer inhibitors. The MMP-2 and MMP-14 (aka MT1-MMP) proteins have been discovered to be present at the forefront of many cancers and can lead to poor prognosis in the patient, due to their ability to migrate to and invade across barriers, eventually leading to metastatic spread.

This study was based on the overexpression of the intracellular variant of MMP-2 (MMP-2ic) and the knockdown of MMP-14 in HSC-3 cells and analyzing for their migratiive and invasive abilities. Although successful overexpression of the MMP-2ic clones was achieved, the knockdown of MMP-14 was not as conclusive and thus migrative and invasive study cannot be concluded upon.

1. INTRODUCTION

1.1 The Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are members of the endopeptidase family that are able to cleave the proteins of the extracellular matrix [1] and other substrates both in normal physiological processes as well as under pathological conditions[2-6]. They are mostly involved in tissue remodeling and have been shown to be conserved across the animal kingdom [7, 8].They can be secreted, membrane-bound or intracelullarly located [9]. In general, they form a family of closely related zinc-dependent endoproteinases. MMP discovery was dated back to the early 1960's, when Gross and Lapiere were trying to ascertain the reason behind the loss of tails in tadpoles during metamorphosis and this led to the discovery of the very first MMP, MMP-1[10]. MMPs are excreted by a wide range of connective tissues which include fibroblasts, endothelial cells, osteoblasts, macrophages, lymphocytes and neutrophils. There are about 23 members of the MMP family which share some specific structural and functional components [11].

In essence, the basic structure of the domain of the members of the MMP family could be said to be consisted of a hydrophobic signal peptide responsible for secretion by guiding the MMP into the RER (rough endoplasmic reticulum) during synthesis; a propeptide domain that maintains the enzyme in its latent form; a catalytic domain consisting of the highly conserved zinc binding site; as well as a hemopexin-like C-terminal domain, PEX, that is connected to the catalytic domain through a flexible region called the hinge region. The zinc binding site in the catalytic center is made up of the HEBXHXBGBXHS motif where H is histidine, E is glutamic acid, B is bulky amino acid, X is variable amino acid and S is serine [12] .The PEX domain is present in most of these MMPs and is

involved in MMP activation through its ability to bind endogenously to TIMPs (Tissue inhibitors of MMPs) and some MMP substrates [5, 11].
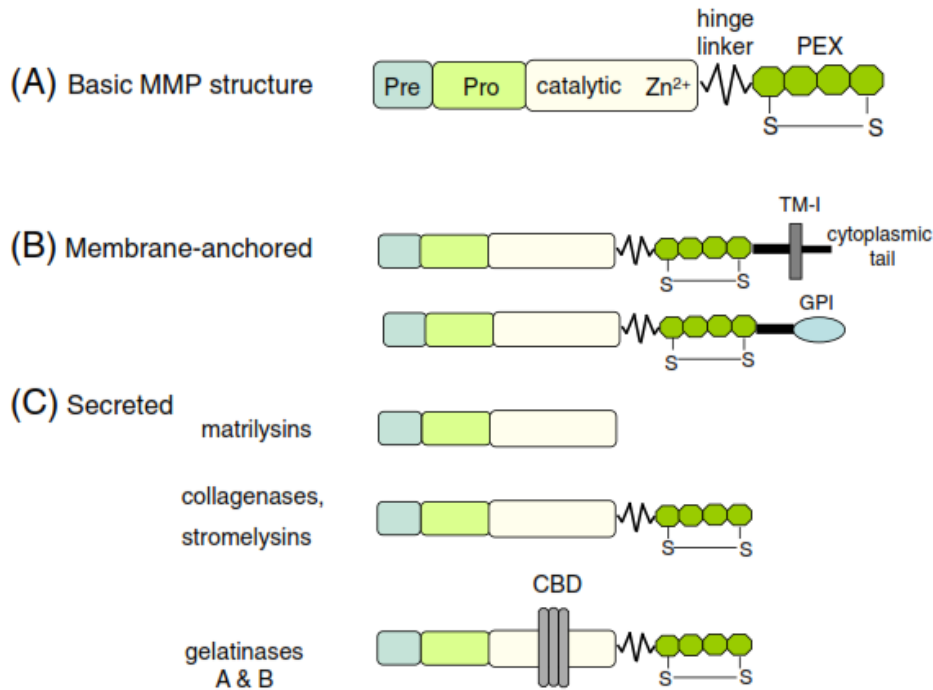
Figure 1: the Basic structure of Matrix Metalloproteinases [11]

MMPs have the ability to degrade both extracellular and non-extracellular matrix proteins and as a result, can influence physiological processes [13] such as bone morphogenesis, early embryogenesis and development, menstrual cycle, wound healing, remodeling processes etc. [14, 15] by the means of their effect on cell proliferation and differentiation, survival, invasion, migration, cell-cell interaction, angiogenesis, inflammation, apoptosis etc. [16, 17].Through their ability to cleave and release cell surface proteins, latent growth factors and cytokines embedded in the extracellular matrix and as a result of their normal physiological functions [11, 18], MMPs are tightly regulated at several points which include activation, transcription, inhibition of the activation and compartmentalization[19, 20]. MMPs have been shown to be expressed in

tumor and neoplastic stromal cells as well as in fibroblasts and inflammatory cells [17, 21]. They have been able to give rise to various pathological conditions as a result of their proteolytic activities and thus, their inherent proteolytic activity and possible dysregulation have been implicated in certain pathological conditions such as arthritis, arterosclerosis, nephritis and even cancers such as acute lymphoblastic leukemia, chronic B lymphocytic leukemia (CLL), acute myeloid leukemia, Hodgkin's and non-Hodgkin's lymphoma etc [11, 22]. MMPs can also be classified by means of the substrates they act on, into Gelatinases (A and B) which degrade gelatin eg MMP-2 and MMP-9; Collagenases, that are able to degrade collagen eg MMP-1, MMP-8 and MMP-13; Stromelysins eg MMP-3, MMP-10 and MMP-11and Matrilysins eg MMP-7 and MMP -25; others include MMP-12 and MMP-26[23, 24] [3, 11].

The gelatinases, occurring as gelatinase A (MMP-2) and gelatinase B (MMP-9), differ from the rest of the MMPs in that they harbour the CBD (collagen binding domain) within their catalytic domain. The catalytic domain in turn, is made up of repeats of fibronectin II type and enables the binding of collagen, elastin, fatty acids, thrombospondins etc [25, 26].

1.2 MMP-2 STRUCTURE AND FUNCTION

MMP-2s, Matrix metalloproteinase-2, AKA gelatinase A, are extracellular matrix proteins and as members of the MMP family, are zinc-dependant endopeptidases which have the ability of cleaving the proteins of the extracellular matrix under normal physiological conditions as well as in pathological condition [3]. The MMP-2 is usually secreted as a 72kDa inactive zymogen, proMMP-2 and its activation occurs when the prodomain is cleaved, giving rise to a 65kDa active form [11]. MMP-2 activation can be achieved by

several processes which include activation by MMPs-1, -7, activated protein C, thombin , MMP-14 interaction as well as autoactivation by collagen α2 VI chain. Out of the above mentioned processes, the proMMP-2 is mostly activated on the cell surface through its complex formation (via its PEX domain) with the MT1-MMP (via its catalytic domain) and TIMP-2 [27] and both MT1-MMP and TIMP have been shown to regulate their activities [28]. Once activated, MMP-2s acquire the ability to degrade/or cleave the components of both extracellular and non-extracellular matrix proteins and this in turn can lead into several processes that can be implicated in both normal physiology and of course pathological processes[29]. Activated MMP-2s are able to promote cell migration, invasion and angiogenesis[30], which in turn promotes tumor progression and metastatic spread[31, 32] by working in concert with the extracellular matrix, growth factors such as chemokines, adhesion molecules and of course the basement membrane[33-36]. It is the actual deterioration of the basement membrane that leads to metastasis. Although MMP-2's were thought to directly degrade the basement membrane, they were later found to perform indirect cell signaling functions by influencing the bioavailabilty and bioactivity of molecules which target specific cell growth, migration, inflammation and angiogenesis receptors [11, 37]. Gelatinases, amongst other things have been the most implicated MMPs in cancer due to the fact that they are usually overexpressed in a lot of malignant tumors and their expression and activities are indicative of the aggressiveness of a tumor [38, 39], as well as a sign of poor prognosis/ survival [40-44]

Although MMPs are basically extracellular proteins, some MMPs such as the MMP-1, MMP-2 and MMP-11 can be intracellularly located and may possibly act on intracellular proteins[4, 9, 27, 33, 45-47]. While much is known about the extracellular mechanisms

of MMP-2, it is not the case with the intracellular mechanisms. However, they have been implicated in cardiac pathologies [46, 48, 49]. The intracellular MMP-2 in the heart muscles have been shown to be directed by oxidative stress in a mechanism that does not require the proteolytic removal of its propeptide [33, 50]. This usually occurs in cardiovascular pathologies such as ischemic reperfusion injury [33, 51]. Acute changes in the myocardial contractile function have also been shown to be caused by the intracellular localization of MMP-2s and their ability to cleave specific proteins necessary for myocardial contractility [9, 50].

The existence of intracellular MMP-2 can be explained by the occurrence of about 3 MMP-2 isoforms [46]. While the first generally known "classical" full length MMP-2 undergoes the "proper" secretion pathway through the translation of a full-length MMP-2 mRNA that gets exported to the extracellular space via secretory vesicles in the latent form [46], there yet exists two others that do not get to enter the extracellular space. This is basically due to the fact that they lack the signal peptide that directs them to the extracellular space and are hence trapped in the intracellular environment. The pathways that have been proposed for their intracellular "entrapment" are as thus: A fraction of the first intracellular variant tends to escape the secretory pathway following its translation and hence localizing to sarcomeric proteins as well as troponin I. In the case of the second intracellular variant, an N-terminal truncated mRNA transcript is generated when a latent promoter in the first intron of the MMP-2 gene is activated during hypoxia and redox stress. The resultant MMP-2 is a 65 kDa MMP-2 isoform lacking the secretory sequence and the inhibitory prodomain which is enzymatically active [46].
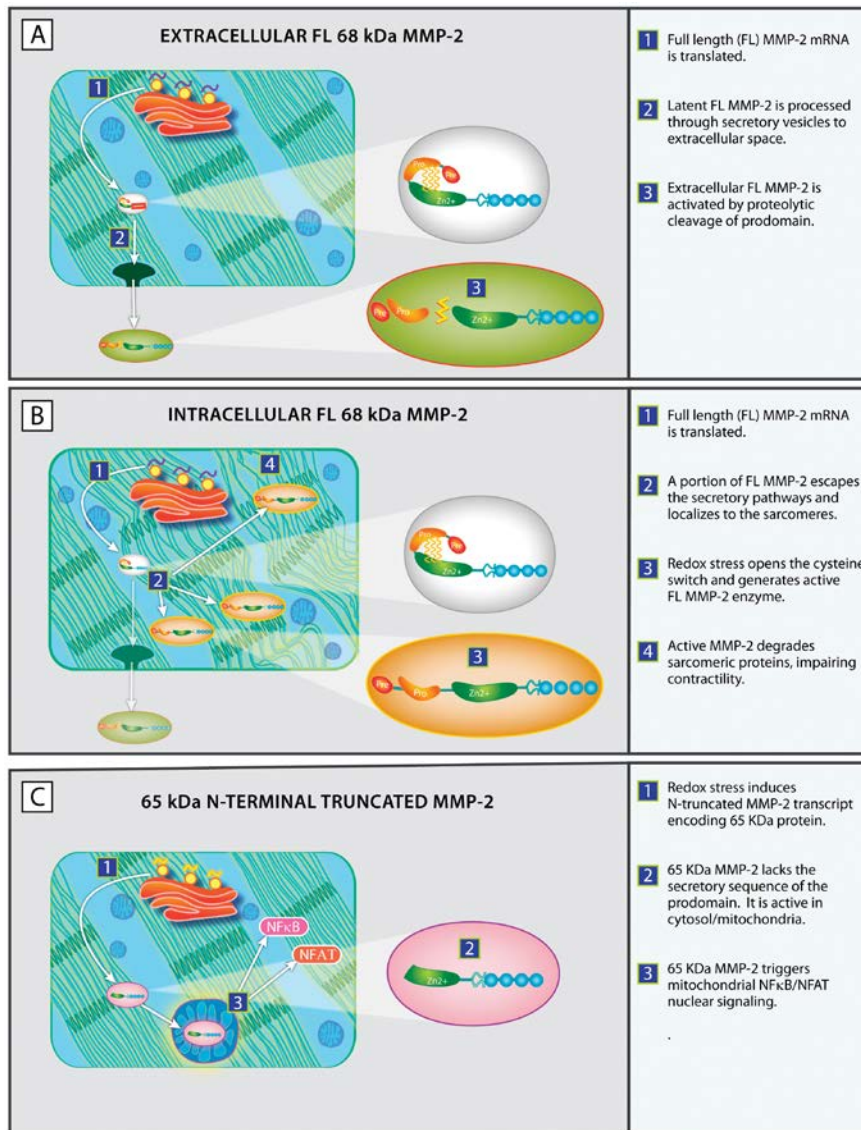
Figure 2: The three MMP-2 isoforms and their processing, localization and activation [46]

1.3 MMP-14 STRUCTURE AND FUNCTION

MMP-14 aka Membrane-type 1 Matrix metalloproteinase (MT1-MMP) are members of the MMP family that are located on the cell surface, being bound to the cell membrane via a TM domain or GPI anchor [11]. While the secreted MMPs such as the gelatinases have repeats of the fibronectin type-II- like domains which interact with gelatin and collagen, the Membrane type MMP (MT-MMP) which consist of 6 members, MT1-MMP

to MT6-MMP, are anchored to the cell surface either through a C-terminal transmembrane domain (TM-1) or the glycosylphosphatidylinositol (GP1) anchor [11]. This makes this group of MMPs unique and in addition to that is the fact that they house a furin recognition motif between their prodomains and their catalytic domain, as well as being processed by proprotein convertases eg furin, in the secretory pathway [11]. They are thus, mostly located outside the cell in active form, where they also carry out their function. The MT1-MMP functions in the activation of pro-MMP-2 and in the degradation of some macromolecules of the extracellular matrix such as collagen I, II and III, laminin-1and laminin-5, fibronectin, vitronectin, fibrin, cartilage proteoglycans, aggrecan and by so doing, promotes cell migration and invasion [52]. MT1-MMPs are inhibited by TIMPs 2-4 and are insensitive to TIMP-1, a feature shared among all MT-MMPs except for MT4-MMP. In the absence of their inhibitor, MT1-MMPs are rapidly autodegraded into a 44kDa form and thus, active MT1-MMPs are usually of very low concentration on cell surfaces as a result of their rapid turnover rate [34].
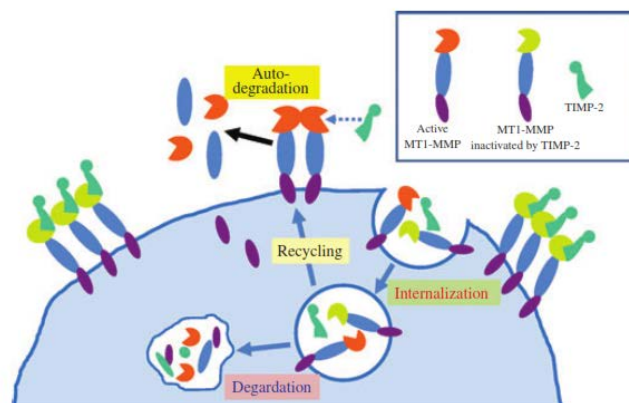
Figure 3:The pathway of MT1-MMP presentation on the cell surface [34]

This in essence explains the occurrence of MT1-MMP in complex with their inhibitor such as TIMP-2 (which is the most common inhibitor) on cell surfaces. The MT1-MMP-TIMP-2 complex, eventually serves as a receptor for pro-MMP-2 on the cell surface.
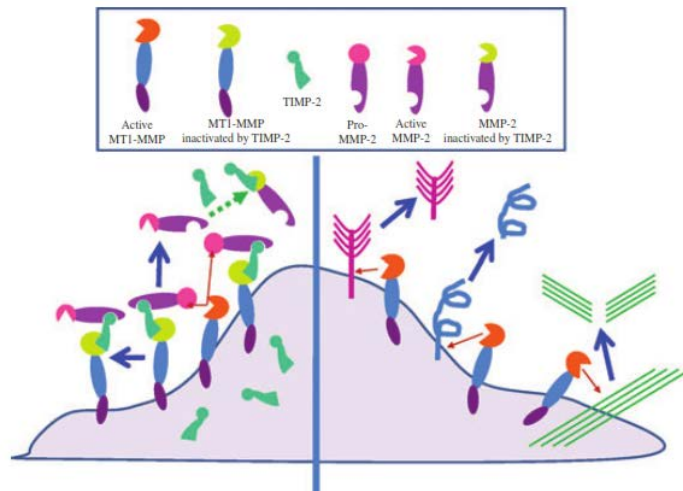
Figure 4: Determination of MT1-MMP substrate choice by TIMP-2 [34]

Collagen has been shown to be the most important substrate that is degraded by the MT1-MMP during the course of cell migration [53], being the most abundant ECM component in the human body and thus posing as a huge barrier to migrating cells. Structurally the collagen is made up of three long left-handed helical polypeptides that are looped with one another in a right-handed configuration [54]. These in turn form polymeric fibrils at 37°C under a neutral pH and are then deposited into the tissue as an insoluble matrix. As a result of its triple helical state, the collagens are mostly resistant to degradation by most proteinases at a neutral pH, but under the same condition, are susceptible to collagenolytic enzymes that are present in the MMP family [54]. The MMP family members that exhibit collagenolytic activities are MMP-1, MMP-8, MMP-13, MMP-2 and MMP-14 (MT1-MMP). Out of these classical collagenases, only the MT1-MMP is membrane-bound and as thus is involved "first-hand" in cell migration and invasion [54].

The extracellular matrix performs several functions in an organism, serving as a glue that enables the holding together of cells, giving structural functions to organs through the provision of framework for tissues, serving as signaling molecules, as well as playing the role of a scaffold that is necessary for the survival, differentiation and functioning of a cell [18, 54]. Thus, while the ECM is a scaffold to migrating cells, it can at the same time act as a migration barrier [54]. As a result of their function in body tissues, the turnover of the ECM is highly regulated since its excessive degradation or lack of it can affect cellular behaviours in particular and as such, give rise to tissue/organ functional defects [18, 55].

MMPs are usually overexpressed in various malignant tumors with a strong correlation established between their overexpression and tumor aggressiveness, tumor stage as well as prognosis [56, 57]. Thus the presence of MMP activity around a tumor is an indication of increased tissue remodeling and degradation. The tumor molecular environment had been shown to be favorable for the activation of MMPs and in fact the observation of activated MMP-2 in various tumors informed the line of thought on the presence of pro-MMP-2 activators in tumor tissues. This line of thought eventually led to the discovery of the MT1-MMP, which was shown to activate pro-MMP-2 on cellular surfaces. MMP-2 and MMP-14 are able to work in concert to promote angiogenesis and metastasis in cancer due to their roles in tissue remodeling via basement membrane degradation [58].

Tumor cell invasion is proposed to undergo a three-step event such as adhesion to ECM structures, proteolytic degradation of the ECM and migration of the tumor cells into the degraded area [56]. In order to mediate tissue remodeling and promote invasiveness in

malignancies, the MMPs would have to be in their active form. Several mechanisms have been shown to activate pro-MMP-2 and these basically occur by the proteolytic cleavage of the prodomain. Examples of these processes include the TIMP-regulated activation by the MT1-MMP, activation by other MT-MMPs and activation through the interaction of the pro-MMP-2 with elastin, heparin and CD151 [55]. The most important of these processes and in reference to the present study is the activation of the pro-MMP-2 by the MT1-MMP-2 which also shows the relationship between the two MMPs in cell migration and invasion. The relationship between the MMP-2 and the MT1-MMP can be seen in its activation and function.

It has been suggested that proMMP-2 can be activated by MT1-MMP in the presence of TIMP-2, by the formation of a ternary complex between the molecules, in which TIMP-2 acts as a bridge between the MT1-MMP and pro-MMP-2[59, 60]. The ternary complex is in such a way that the N-terminal region of the TIMP-2 is linked to the MT1-MMP, while its C-terminal region is linked to the hemopexin region of the proMMP-2. In order to finally activate the pro-MMP-2, there is the need for another MT1-MMP to cleave it, giving rise to an intermediate 64 kDa molecule that eventually becomes processed into the fully active 62 kDa form by autocatalysis [34]. The net activity of MT1-MMP and MMP-2 is regulated by the concentration of TIMP-2 and other inhibitors in a complex manner [34, 42]. Other TIMPs such as TIMPs-1, -3 and -4 are also able to regulate the activation of pro-MMP-2 via MT1-MMP and TIMP-1 has been shown to keep the pro-MMP-2 in its inactive intermediate form, thus preventing its autoactivation.

Following their activation by MT1-MMP, MMP-2 acts by breaking down tissue and bringing about remodeling during tumor invasion, they get intravasated into the blood

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circulation, they become extravasated and migrate to sites of metastasis by promoting angiogenesis [56]. While MMP-9 has been initially thought to be the most important MMP in cell invasion and metastasis as a result of their overexpression in cancer cells as well as their induction by numerous cytokines, growth factors and products of oncogene, MMP-2 has been shown to be involved in a lot of cancers [61]. There has been a suggestion that an increase in MMP-2 activity actually contributes to the very first step in metastatic spread [62] [38], through the promotion of angiogenesis [63], tumor cell infiltration across the basement membrane as well as invasion into the circulation [64, 65]. Invasiveness of both normal and neoplastic cells has been shown to be promoted by MMP-2 and it has been shown that pro-MMP-2 that are overexpressed in either serum or by stromal fibroblasts in cancer tissues are somewhat activated on the cancer cells by the MT1-MMP. Thus a correlation has been made between the overexpression of MMP-2 to that of MT1-MMP in the presence of TIMP-2 [66].

1.4 MMP-2 and MMP-14 in Cell Migration and Invasion

Cell migration and invasion are complex processes involving the ECM, proteinases, chemokines, adhesion receptors and the basement membrane [67]. MMPs play significant roles under normal physiological condition part of which includes wound healing, in which there need for the migration, proliferation, ECM disposition as well as remodeling [68, 69]. This process enables the recruitment of immune cells and growth factors to the site of injury [70].
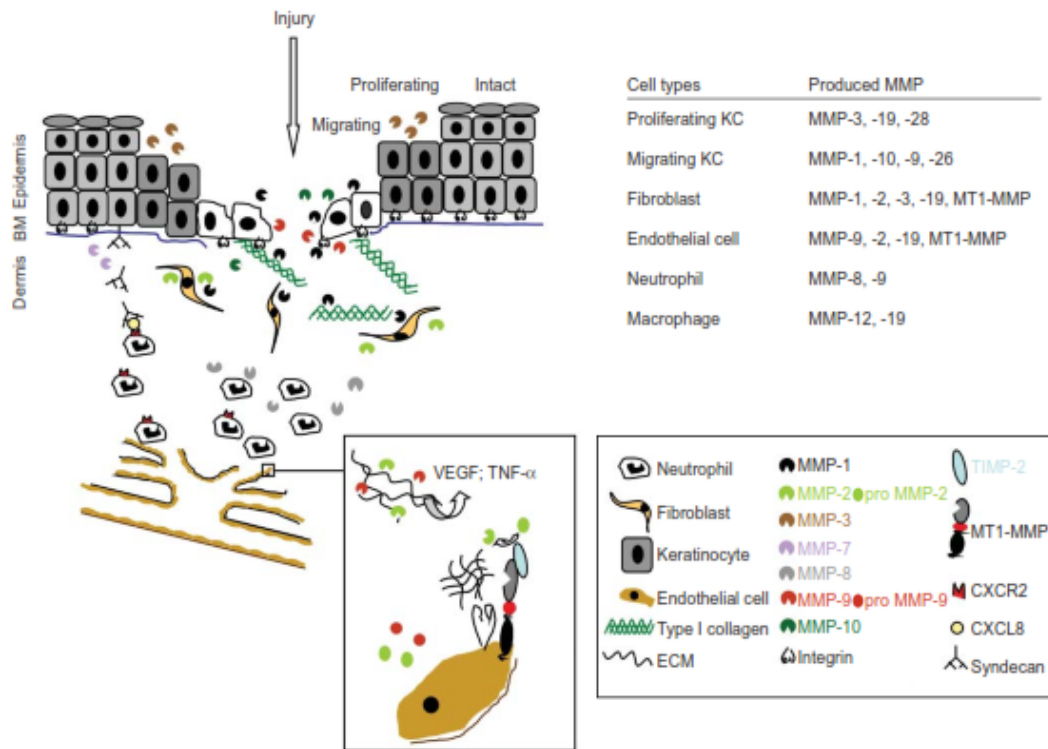
Figure 5: MMPS in wound healing and angiogenesis [20]

In pathology, it seems like the same mechanism as that in wound healing seems to be employed to facilitate the invasion of tumor cells from the primary tumor site to metastatic sites [69, 71-73].Thus the basic promigratory machinery that are employed in normal cells happen to be retained in tumor cells and their migration seems to be activated by the heightening of promigratory events in the absence of counteracting stop signals [67, 72, 74]. The imbalance of these signals enables the continuous migration and invasion of the cancer cells, causing the tumor cells to expand across tissue barriers and eventually leading up to metastasis [72]. The presence of MT1-MMPs in tumor cell lines have been shown to be a driving factor in the penetration of the basement membrane as well as in the invasion of cells through the interstitial type-1collagen tissues [60]. MT1-MMP are known to be accumulated at specialized ECM-degrading membrane protrusions on invasive cells known as the invadopodia [52, 71, 75] and in

addition to the MMP-2, have been found to be present in high amounts at tumor invasive fronts in which they breakdown the matrix [38, 54, 69].
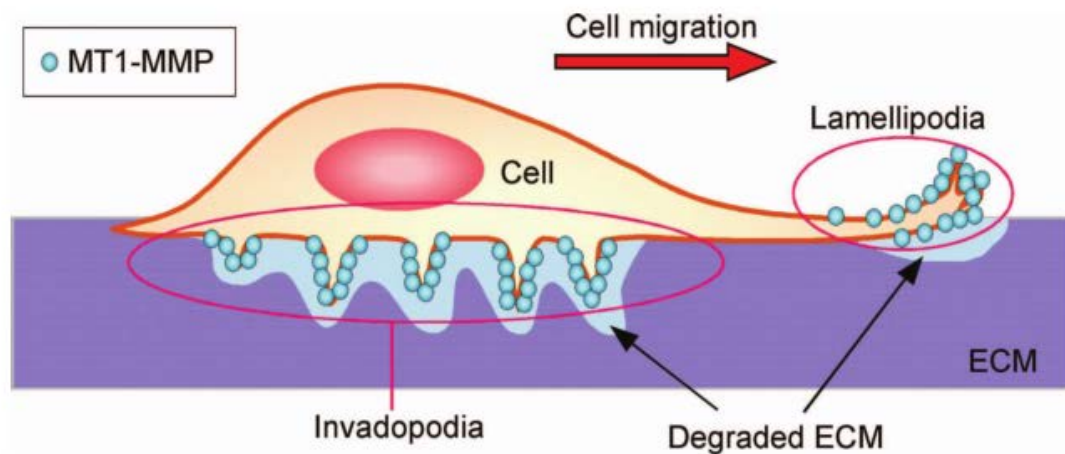
Figure 6: MT1-MMP and Invadopodia and Lamellipodis formation for cell migration [54]

As the most common physiological activator of the MMP-2, a deficiency in MT1-MMP has been seen to lead to the production of very minute levels of active MMP-2 in mice [34]. Again, while a knock-out of MMP-2 in mice has led to phenotypic changes such as small stature, blunted tumour-related angiogenesis and abnormal alveoli in the lungs; a knock-out of MT1-MMP has been associated with highly defective angiogenesis, skeletal development and eventually death shortly after birth, around a couple of weeks [34, 76]. This is an indication of the level of importance of this MMP. Worse still is the effect of the double-knockout of both MMPs, in which the mice were born with abnormal blood vessels, immature muscle fibres and respiratory failure, dying as soon as they are born [14, 34]. This again goes to show that though these MMPs are important in the maintenance of homeostasis, they are on the other hand implicated in pathology such as their involvement in tumor growth and in the multistep processes of invasion and metastasis [77].

As cancer cells migrate/invade, there is the need for the formation of new blood vessels (angiogenesis) which enhances tumor growth and metastasis [53, 78, 79]. Under normal physiological conditions, the development/establishment of vascular supply and modelling [2] is of critical importance for growth, maturation and tissue maintenance [30] and this happens to be the case with metastasis [80], where the primary tumor establishes its own blood supply derived from the host cell [30]. Vascular proliferation and vessel formation are influenced by certain factors such as acidic and basic fibroblast growth factor, TGF and TNF alpha [29]. Although the MMPs are directly involved in tissue remodelling, they have been shown to also carry out this function through their interaction with cytokines and growth factors [35, 79].Through their degradative impact on the ECM, the gelatinases tend to release or generate bioactive molecules that in turn influence the growth of the tumor [35] [81]. This can be seen as the release of cryptic/hidden information from the ECM and basement membrane that can give rise to the migration of cells and thus, metastasis [11].

In the case of metastatic breast cancer for example, the migration of breast epithelial cells has been shown to be induced by MMP-2 activity in which case the MMP-2 cleaves and regulates the function of laminin-5 (Ln-5), which is a component of the ECM [22, 61]. Cells have the tendency to attach to the ECM substrates with the help of the integrin receptors and the effect of the gelatinases and plasmin on selected components of the basal membrane, shows that activated gelatinase cleaves the Ln-5 subunit at residue 587, exposing a possible pro-migratory cryptic site which is able to provoke the motility of cells as against adhesion. On the other hand, the cleavage has the potential to cover up a site that inhibits cell motility. As a result of the fact that pro-migratory cryptic sites do not promote adhesion indicates the influence of MMP-2 proteolytic activity in the provision of signaling mechanisms to initiate cell migration in mammary gland

morphogenesis [22]. This was seen as the case in the enhanced migratory response to gelatinase A-modified Ln-5 in MCF-7, which is a neoplastic epithelial cell line [22, 82].

Although MMP-2 is generally known to exert its function in the extracellular environment, this project was aimed at looking at the intracellular subtype of MMP-2 (MMP-2-ic) and its possible role in the migration and invasion of tumor cells since it has been shown to be expressed in cancer cells. For this purpose, oral squamous cell carcinoma cell line, HSC-3, was used to overexpress the intracellular MMP-2, since it was not expressing either the extracellular or intracellular MMP-2. The same cell line, that has been shown to be expressing MMP-14 (MT1-MMP) was knocked-down for MMP-14 after which the effects of the MMP-2 overexpression and the MMP-14 knockdown was analysed using the xCELLigence assay.

At the start of the experiment, the expression levels of MMP-14 and MMP-2 was determined in wild-type HSC-3 cells (HSC-3wt) using the western blotting and SDS-PAGE gelatin zymography techniques in both cell lysates and conditioned medium.

The cells were then overexpressed for Intracellular MMP-2 (MMP-2ic) by first determining the optimal concentration of the G418 antibiotic that was required to kill the HSC-3 cells, followed by the transformation of the DH5α competent bacteria with the pM02-MMP2ic (Z5731) and pReceiver-M02 (empty vector) plasmids respectively . The colonies expressing the plasmids were selected on LB plates with ampicillin followed by purification and verification of the plasmid insert by sequencing and restriction enzyme cleavage after which they were used to stably transfect the HSC-3 cells using G418 as the stable selection marker.

The overexpression of MMP-2ic was confirmed by western blotting for the determination of the protein and RNA transcript respectively, using two colonies respectively from the EV transfectant and the overexpression transfectant. For the MMP-14 knockdown, the E.coli DH5α competent bacteria was first transformed using 4 MMP-14 shRNA constructs including a scrambled control. Colony selection was done on LB medium plates (with 100μg/ml ampicillin) followed by plasmid purification. The concentration and purity of the purified plasmid was determined on the Nanodrop after which the plasmids were used to transiently transfect HSC-3 cells for MMP-14 knockdown out of which the construct that gave the best knockdown was used for stable transfection, to select for mixed bulk clones expressing mMP-14 knockdown with puromycin as the stable selection marker.

Eventually, all the MMP-2 and MMP-14 cell clones were analysed for subcellular location by immunofluorescent staining and confocal microscopy; in situ zymography was employed on the FL-MMP-2 and EV control to analyse for gelatinolytic activity and finally, the HSC-3-EV, HSC-3-MMP-2ic, HSC-3-sc and HSC-3-MMP-14kd clones were compared in terms of cell migration and matrigel invasion, using the xCELLigence system.

1.5 The shRNA Concept

shRNAs are short hairpin RNAs that are utilized in the RNA interference technology to bring about a knockdown/silencing of a gene, by targeting their mRNA. They are sequences of RNA (19-22nt) capable of making a tight hair-loop structure (4-11), similar to those in miRNA, and have the ability to degrade target RNA [83]. The in vitro expression of shRNA in mammalian cells is made possible through direct plasmid delivery, use of bacterial or viral vectors and the choice of method is dependent on the

cell's "transfectability" [84]. The use of viral vectors (infection) has been associated with a much more successful knockdown of the target gene due to the long-term integration into the host genome [84]. The shRNAs are transcribed in the nucleus by either RNA pol II or modified polymerase III promoters of the expression vector that bears a short double stranded DNA sequence containing a hairpin loop and following their transcription, get exported to the cytosol where they get recognized by Dicer, an endogenous enzyme that cleaves them into duplexes of siRNA [83]. This endogenously-derived siRNA oligonucleotide functions in the same manner as the exogenously-derived siRNA by binding to the target mRNA and loaded onto the RISC complex for target-specific mRNA degradation [83], translational inhibition and heterochromatin formation [85-87].

1.6 The Principle of Gelatin Zymography

The use of SDS-based Gelatin Zymography involves the use of denaturing, non-reducing conditions (SDS) for the separation and detection of proteins (gelatinases) [88-90].

Usually, SDS polyacrylamide gel electrophoresis is used to separate electrically charges molecules moving against an electric field in such a way that smaller molecules move more slowly than the larger ones. This is made possible when the anionic detergent in the protein sample preparation buffer, SDS, reacts with the polypeptide chains of the protein causing their linearization as well as imparting them with negative charges. The charges are evenly distributed per unit mass and this eventually results in their near exact separation on the polyacrylamide gel during electrophoresis.

The gelatin chromatography technique is only different from the SDS-PAGE in the sense that gelatin is incorporated into the separating gel in which it co-polymerizes with the

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acrylamide [91]. The separating gel thus functions as a matrix that allows for the detection of gelatinases through their degradation of the gelatin. During the electrophoresis, there is a denaturation of the MMP by the SDS and this gets partially renatured and activated during the washing step when the SDS is exchanged for Triton®-X100; the latent MMPs also get autoactivated at this stage without any cleavage. Eventually, during incubation in the appropriate incubation buffer, the concentrated and renatured MMPs digest the gelatin and the digestion can be detected as clear bands on blue background (undigested substrate) following staining with Coomassie blue. Gelatin zymography is a very sensitive and reliable technique for the detection of the gelatinases, detecting as little as 10pg amounts [4]. That notwithstanding, other MMPs such as MMPs-1,-8 and -13, but this could appear as very faint bands which become stronger when separation is done with the appropriate substrates for these MMPs such as casein and collagen.

1.7 In situ Zymography

In situ zymography is a method that can be employed in the detection and localization of enzymatic activity within cell culture or tissue sections [4, 21, 89, 92-94]. As a result of this, the enzyme in question has to be in its active form especially since they are secreted in their inactive pro-form and thus, this method detects only the active form of the enzyme. This gives a clear difference between the in situ zymographic technique as against that of the gelatin zymography in the sense that while the gelatin zymography is used in the detection of all forms of the enzymes such as pro- and active, the in situ method is more discriminatory with its affinity for only the active form.

There are 3 types of in situ zymography:

- The photographic emulsion-based method in which tissue sections are immersed in silver-coated substrate containing photographic emulsion in which light microscopy is used in the detection of proteolytic activity. Thus substrate cleavage is detected as reduction in silver concentration which appear as white patches on a dark background [91].

- The fluorescently labelled method involves the coating of glass slides with fluorescent substrate followed by the mounting of tissue sections onto the slides and incubation. Detection is done under fluorescent microscopy in which proteolytic activity is observed as dark patches on a fluorescent background [91].

- The third method, the highly quenched substrate method, was used for this project due to its high sensitivity and ease of standardization. It involves the incubation of tissue sections or cell culture on glass slides, with a highly quenched fluorescently labelled substrate (DQ gelatin) and following fluorescent microscopy, proteolytic activity is detected as fluorescence against a dark background [91].

Although ISZ has the advantage of determining the proteolytic activity of only the active enzyme in question, it has a disadvantage in that it measures the net activity of all the proteases that degrade the substrate. In essence, the specificity of the enzyme(s) for the substrate determines to a large extent, the measured enzyme activity and since most substrates have a large mileu of proteases, pinpointing the exact protease responsible for the enzyme activity can pose a difficulty. This can be controlled by the combination of ISZ with IHC or in situ hybridization, incorporation of inhibitors eg EDTA, specific inhibitory peptides [94], TIMPs, small molecule inhibitors as well as monoclonal antibodies.

1.8 xCELLigence

The xCELLigence system uses the readout of impedance to noninvasively quantify the status of cells in real-time [95, 96]. This makes it possible to assess cell behavior such as adhesion, proliferation, migration, invasion as well as cytotoxicity, without any disruption to its natural environment [95, 97-101].This method has the advantage of enabling a continuous quality control of cells [96, 102]. The RTCA-DP instrument is made up of two components; the RTCA Control Unit and the RTCA DP Analyzer, with three stations which are integrated for the measurement of cell responses either individually or in parallel. The xCELLigence system makes use of two types of impedance-based 16-well plates such as the E-16 plate/E-Plate VIEW (for cellular assays) and the CIM-PLATE 16 (for migration and invasion assays). The plates are constructed in such a way that they are made up of a top and a bottom chamber. Beneath the top chamber are gold-plated microelectrode sensors.

The cells are seeded on the top chamber of the plate and are able to move through the microporous membrane (of the TC) into the bottom chamber containing a chemoattractant such as FBS. The RTCA DP Analyzer and the plates containing the cells are placed in a CO_2 incubator to attain normal cell culture conditions. The adherence of cells to the microelectrode sensors leads to an increase in impedance which gets read-off in real time by the xCELLigence RTCA DP instrument [97, 98, 102, 103]. The impedance readout is usually affected by several factors such as seeded cell number, adhesion and morphology and thus different cell lines will exhibit different proliferative patterns depending on the density seeded. Again, when cells are treated with cytotoxic compounds, each of the above features is uniquely affected, yielding unique Time

Dependent Cellular Response Profiles. The resulting response profiles will then be able to aid in the optimization of experimental conditions such as seeding densities and time; they would made the elucidation of a compound's mechanism of action possible; and perhaps aid in comparative analysis [97, 98].

2. MATERIALS AND METHODS

2.1 List of Materials

CELL CULTURE MEDIA	Dulbecco's Modified Eagle Medium: Nutrient Mixture-F12 (DMEM-F12)	#31330-038. Gibco®Life Technologies, Carlsbad, CA
	OPTIMEM	#31985-047. Gibco®Life Technologies, Carlsbad, CA
	Fetal bovine serum (FBS)	#F7524. Sigma-Aldrich, Oslo, Norway
	Luria Bertani Broth (LB Broth)	10g Tryptone 5g Yeast Extract 10g NaCl Make up to 1 litre with Milli-Q water and autoclave
	Super Optical broth with Catabolite repression (SOC) medium	20g Tryptone 5g Yeast Extract 3.6g Glucose 10mM NaCl 2.5mM KCL 10mM $MgCl_2$ 10mM $MgSO_4$ Make up to 1 litre and autoclave
CHEMICALS /REAGENTS	1X Trypsin-EDTA	#T3924. 0.5% Trypsin, 0.2g/L EDTA. Sigma-Aldrich, Oslo, Norway
	Freezing Solution (Cells)	FBS + 10% DMSO
	Freezing Solution (Cell supernatant)	1M Hepes (pH 7.4) + 0.1M $CaCl_2$
	Hydrocortisone	# H6909. Sigma-Aldrich, Oslo, Norway
	Ascorbic acid	# A7506. Sigma-Aldrich, Oslo, Norway

	Lipofectamine-2000	Cat # 11668-027.Invitrogen Life Technologies, New-York, U.S.A
	Prestained ladder	P/N 100006636. Invitrogen, Carlsbad, CA
	Magic marker	Lot # 1030592 P/N LC5602. Invitrogen, Carlsbad, CA
	Biotin ladder	#7716S. New England BioLabs, Ipswich,U.K
	1kb ladder	# SM13311.Fermentas ladder Gene Ruler 1kb DNA ladder Plus. Thermo scientific, Oslo, Norway
	100bp ladder	Fermentas Gene Ruler™100bp DNA ladder # SM 0321
	SYBR Green	#330523. Qiagen, Hilden, Germany
	BSA	#A9647. Sigma-Aldrich, Oslo, Norway
	BioRad Dc Protein AssayKit	Reagent A: Cat # 500-0113 Reagent B: Cat # 500-0114 Reagent S: Cat # 500-0115 Hercules, California
	Qiagen QIAprep®Spin Miniprep kit	#27106. Qiagen, Hilden, Germany
	Nucleobond® Xtra Midi	# 740 410 50. Macherey-Nagel, Duren, Germany
	Quantitect Reverse Transcription kit	# 205313. Qiagen, Hilden, Germany
	Nde 1 Restriction enzyme	Nde 1 R0111S.Ipswich, U.K
	Goat Serum	Dako Lot # 00095732 code X0907. Glostrup, Denmark

	DQ Gelatin	#D12054. Invitrogen, Carlsbad, CA
	Matrigel	# 356234. New Jersey, USA
BUFFERS AND SOLUTIONS	4% PFA	0.4g PFA 9ml Milli-Q water Adjust pH to 7.5 1M NaOH 1ml 10X PBS
	Cell lysis buffer	0.1M Hepes 10mM $CaCl_2$ 1% Triton
	RIPA Buffer	25 mM Tris HCL pH 7.6 150mM NaCl 1% Triton x-100 0.5% Sodium deoxycholate 0.1% SDS Protease inhibitor (100µl/ml buffer) ☑ Sigmafast™ Protease inhibitor cocktail tablets, EDTA free (S8830)
	RNA PURIFICATION	
	Cell Lysis Buffer	350µl RLT Buffer 6 µl 3M DTT
	• Buffer RLT	Lot # 142319059
	RNeasy kit	# 74134
	QIA shredder	#79654
	GELATIN CHROMATOGRAPHY	
	Equilibration Buffer	0.1M Hepes pH 7.5 0.05% Bri
	Washing Buffer	0.1M Hepes pH 7.5 0.05% Bri 0.5M NaCl
	Elution Buffer	0.1M Hepes pH7.5 0.05% Bri 0.5% DMSO
	WESTERN BLOTTING	
	1x NuPAGE Running	30µl 20x MES SDS

	Buffer (MES SDS)	570 Milli-Q water
	1xTBST	60ml 2.5M NaCl 20ml 1M Tris pH 7.4 1ml Tween 20 Make up to 1 litre with Milli-Q water
	Blocking Buffer	5% skim milk powder in TBST 1% BSA
	Blotting Buffer	5.8g Tris (base) 29g glycin 800ml MilliQ-water 200ml Methanol
	NUPAGE Sample Buffer	NP0007 Invitrogen
	Luminol Reagent	Santa Cruz SC-2048.Immunocruz
	Stripping solution	Millipore® Reblot plus solution 10X (2502) 92590
	RESTRICTION ENZYME DIGESTION	
	10x Buffer	NEB Buffer 2 # B70025
	GELATIN ZYMOGRAPHY	
	Electrophoresis buffer pH 8.3 (10X)	30 g Tris base 144g glycine 10g SDS (1% final concentration) + up to 1 litre with Milli-Q water pH adjustment to 8.3 with conc HCL
	Separating gel buffer (0.5M Tris)	0.605g Tris base 0.04g SDS Milli-Q water to 10ml pH adjustment to 6.8 with conc HCL +100µl sodium azide
	Stacking gel buffer (1.5M Tris)	18.2g Tris base 0.4g SDS

		Milli-Q water up to 100ml pH adjustment to 8.8 with conc HCL + 100μl sodium azide
	Washing buffer	10 ml Triton X-100 Up to 400ml Milli-Q water
	Coomassie blue solution	0.4g Coomassie brilliant blue 120ml Methanol Milli-Q 80ml
	Incubation buffer	12.1g Tris base 63.0g Tris HCL 117g NaCl 7.4g $CaCl_2 . 2H_2O$ Brij 35 0.2% Up to 1 litre withMilli-Q water
	Staining solution	25ml 0.2% C Coomassie blue 25ml 20% HAC
	Destaining solution	150ml Methanol 50ml 100% acetic acid 300ml Milli-Q water
	TE buffer	Marligen Biosciences® # 043-70271FT
	50X TAE Buffer	242g Tris 100ml 0.5M EDTA 57ml Glacial acetic acid Up to 1 litre with Milli-Q water
	Biotium Gel Red Nucleic acid stain	Lot # 11G0810
	6X loading buffer	Fermentas #R0611
	4X LDS Sample Buffer	Invitrogen NUPAGE® #NP0007
ANTIBODIES	Anti - human MMP-2 antibody	R&D AF902
	Monoclonal anti goat/sheep IgG	#A9452. Sigma-Aldrich, Oslo, Norway

	Anti-MMP-14 antibody	Novus EP1264Y
	Goat anti-rabbit IgG (H+L chain specific)	Southern Biotech
	Anti- β-actin	A3854. Sigma-Aldrich, Oslo, Norway
	Anti- Biotin	Cell Signalling Tech ® #7075
	IMMUNOSTAINING	
	MMP-2 primary antibody	Abcam # ab37150
	MMP-14 primary antibody	Novus # NB 110-57216
	Secondary antibody (Alexa Fluor® 488 Goat anti-rabbit)	Life Technologies® #A11034
ANTIBIOTICS	G418 (Geniticin)	#G8168. Sigma-Aldrich, Oslo, Norway
	Puromycin dihychloride	#P9620. Sigma-Aldrich, Oslo, Norway
PLASMIDS	EX-Z5731-M02 pReceiver-M02-EX-NEG-M02 shRNA HSH011268-1-nU6 shRNA HSH011268-2-nU6 shRNA HSH011268-3-nU6 shRNA HSH011268-4-nU6	GeneCopoeia. Rockville, USA GeneCopoeia. Rockville, USA GeneCopoeia. Rockville, USA GeneCopoeia. Rockville, USA GeneCopoeia. Rockville, USA GeneCopoeia. Rockville, USA
	GFP	pmax GFP 0049-08
PRIMERS	GAPDH (HS_GAPDH_2_SG)	Qiagen # QT01192646
	ACTIN (HS_ACTB_1_SG)	Qiagen #QT00095431

	MMP-2 (HS_MMP2_1_SG)	Qiagen # QT00088396
	MMP-9 (HS_MMP9_1_SG)	Qiagen # QT00090040
	MMP-14 (HS_MMP_1_SG)	Qiagen # QT00001533
	Sequencing Primer (MMP-2)	Fwd: SY 130826294-056 Rev: SY 130825588-036

2.2 Cell Culture

2.2.1 Thawing cells from Liquid nitrogen

Mammalian cells are usually stored in liquid nitrogen for preservation and in order to re-passage them, they need to be resuscitated. About 5 ml of the appropriate cell culture medium (DMEM-F12 + supplements) was placed in the small cell culture flask (T25), after which the cell vials were taken up from liquid nitrogen, quick-thawed by rubbing gently. As soon as the cells were thawed, they were quickly re-suspended in the cell culture flask, swirled to distribute evenly and incubated in the CO_2 incubator for attachment. Following attachment, the medium was changed to further dilute the DMSO in the freeze-medium.

2.2.2 Splitting/passaging of cells

Cell passaging is done to maintain the cells in their proliferative state otherwise the cell culture tends to become too confluent and the cells begin to die due to the exhaustion of nutrients.

Following 80-90% confluency, the cells were split by aspirating the medium, washing with pre-warmed PBS and trypsinized using 1X trypsin- EDTA. The flasks were then incubated for 4-8 minutes in the incubator, until the cells detached from the flasks. The trypsinization reaction was often stopped by the addition of 4ml medium (for T25 flasks). The cells were then mixed by pipetting gently, after which the desired volume was added into a fresh flask, with fresh medium used to top-up for the appropriate cell concentration. The cells were evenly distributed by sliding the flask upwards, downwards and sideways, viewed under the microscope to check for their distribution and then incubated in the CO_2 incubator at 37°C.

2.2.3 Cryopreservation of Mammalian cells

Cell cultures were frozen down in order to maintain cells with low passage numbers and to limit genetic variations that could be introduced through high passaging rates.

At about 70-90% confluence, the cells were washed with PBS and gently detached by trypsinization and resuspended in the appropriate amount of complete growth medium. The cells were then centrifuged after which the supernatant was withdrawn, leaving behind the pellets. The pellets were loosened by gently flipping the tube after which the cells were resuspended in aseptically prepared and sterile-filtered freezing medium (FBS +10% DMSO) to a final concentration of 5 $x10^6$ to $1x10^7$ cells per ml (approximately 4-6 ml). Aliquots of 1 ml were made into cryogenic tubes after which they were placed in insulated vials and gradually frozen for 1 hour at -20ºC, overnight at -70ºC and infinity (∞) in liquid nitrogen.

2.2.4 Cell counting: Naubauer Chamber

Cell counting is necessary in order to adhere to the required experimental starting cell number.

Cells were grown to confluency after which the media was aspirated, followed by washing the cells in PBS and then trypsinization with 1X trypsin EDTA. The trypsinization reaction was stopped with 4, 8 or 12ml media, depending on the flask size for T25, 75 or 175 respectively

Approximately 10μl of the cell suspension was introduced into the v-sections of the coverslip-naubauer chamber interphase after which the cells were counted under the microscope by focusing the counting grid under low magnification objective.

2.2.5 Cell Harvesting

Cells are usually harvested for preservation or in order to carry out further analysis.

Confluent cells were harvested by first aspirating the medium followed by washing the cells twice in 1X PBS. The cells were scraped with the appropriate lysis medium, incubated on ice for 15 minutes after which they were centrifuged at 9700 rpm for 10 minutes. The cell supernatant were transferred to fresh tubes and frozen at -70ºC.

2.2.6 Gelatin Column Chromatography

Gelatin column chromatography is used for the purification of MMPs by using gelatin sepharose affinity media (containing immobilized gelatin) due to the high specificity with which gelatin binds to the fibronectin-domain in the MMPs.

The columns were washed /equilibrated 3-4 times with 500μl washing/equilibration buffer (0.1 M Hepes pH 7.5, 0.05% Brij) to stabilize the enzyme, after which the samples (from cell lysates or cell supernatant) was applied into the column, 100μl at a time. The

column was then washed twice with 500μl wash solution with salt (0.1 M Hepes pH 7.5, 0.05% Brij and 0.5% NaCl) to eliminate unspecific binding, as well as twice with 500μl wash buffer (with Brij) to get rid of the salt so it does not get into the eluent. To elute, the column was first washed with 30μl of the elution buffer (0.1M Hepes 0.05% Brij and 0.5% DMSO) and finally eluted into new tubes with about 75μl of the elution buffer.

2.2.7 Measurement of Protein Concentration

Protein concentration measurement was necessary to ensure that the required amount of protein was loaded on gels to make them detectable. It is also of importance as a loading control for quantitative analysis. Several techniques are employed in protein concentration measurement and in the course of the project, two methods were used which included the Millipore Direct Detect method and the BioRad DC protein assay method.

2.2.7-1 Millipore Direct Detect Method

Protein concentration measurement by the Millipore Direct Detect spectrophotometer provides protein concentration by measuring the intensity of the amide 1 band. The amide 1 band is assigned to the C=O stretching vibration which accounts for 80%, and about 20% slight contribution by the C-N stretching vibration.

The membrane of the Direct Detect card was loaded with 2μl of each of the cell lysate extracts/ conditioned medium as well as the lysis buffer.

The sample card was then inserted into the Direct Detect machine for the measurement of the protein concentration which is read-off on the machine in real time.

The BioRad protein assay is a colorimetric assay that determines the concentration of proteins through the solubilzation of detergent. Protein concentration is usually obtained when the protein interacts with an alkaline copper tartrate solution which in turn reduces the fiolin reagent, giving rise to differential colour changes which is mostly due to the amino acids tyrosine and tryptophan and to a lesser extent on cysteine, cysteine and histidine. The proteins effect colour change by the loss of 1, 2 or 3 oxygen atoms hence producing one or more of the reduced species one of which one the characteristic blue colour.

Using a 96-well plate, a standard curve assay was prepared using BSA concentrations of 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, 1.0mg/ml, 1.2mg/ml and 1.4mg/ml, for a total of 5µl per well.

5µl of each of the samples was pipetted in duplicates into respective wells after which 25µl per well working reagent (made up of Reagent A and Reagent S in the ratio of 1:50) was added into the standard curve and the sample wells.

200 µl of Reagent B was added to all the wells, followed by 15 minutes incubation at room temperature and reading on the Molecular devices VERSA$_{max}$ tunable microplate reader.

2.3 KILL CURVE

The kill curve experiment was performed prior to the stable transfection of cells for the selection of cell clones. The idea behind it is to incubate cells in different concentrations of the antibiotic in question, after which the antibiotic concentration that will yield the

least cell population of cells after a period of time, will be used for the selection . When this is determined, the same concentration is added to the medium for the stable selection of cell clones. Using the same starting cell population, eg approximately 0.3×10^6 cells per well of a 6-well selective plate, different antibiotic concentrations (starting from 0µg/ml for no antibiotic), were added to the respective wells. The volume was made up to 2ml (for a 6-well plate) after which the cells were incubated for approximately 7 days during which the medium was replaced every 3 days. After about 7days, the antibody concentration that yielded no cells corresponded to the right concentration for the selection.

2.4 MAMMALIAN CELL TRANSFECTION

Mammalian cell transfection is a process whereby exogenous DNA or RNA is introduced in host cell lines.

2.4.1 Transient Transfection

Transient Transfection enables for the expression of exogenous DNA or RNA in a host cell line for short term purposes, usually between 24 -72 hours. In this case, the nucleic acid is not incorporated into the host genome.

On the first day of the experiment, approximately 0.2×10^6 cells were seeded in a 6-well plate and incubated overnight at 37 ºC.

For the transfection reactions (in 6 well-plate), the volume of lipofectamine was scaled-up to 10µl lipofectamine and 90µl optimem, for a total of 100µl. Thus, 10µl of Lipofectamine-2000 reagent was diluted in 90µl of optimem reagent per sample (mix 1)

and in separate eppendorf tubes, 2.5μg of each plasmid was diluted in optimem, to a final volume of 100μl.

100μl of mix 1 was added to each of mix 2 (for the respective plasmids) and then incubated for 5 minutes at room temperature, after which the complex was added to the respective wells and incubated for 48 hours at 37º C. GFP was included as a control, to check for the transfection efficiency. After about 48hours incubation, the transfection efficiency was determined by fluorescent microscopy and then harvested for further analysis by western blotting, RT-qPCR etc.

2.4.2 Stable Transfection (Mixed clone selection)

Stable transfection is a "long-term"/permanent expression of exogenous DNA into a host cell line, with the nucleic acid being incorporated into the host genome.

For the stable transfection, the cells were seeded in T25 flasks on the first day of the experiment and then transfected on the second day when they were about 60-70% confluent. The transfection was performed in the same way as the transient transfection, however, the experimental conditions such as the plasmid amount, lipofectamine and optimem were stepped up according to the protocol. For the T25 flask, the plasmid amount was increased to 6.25μg. Again, the medium was changed every third day post-transfecton with medium containing the appropriate concentration of antibiotic for the selection. Following confluency, the cells were trypsinized and seeded in a T75 flask, for expansion, after which they were frozen down at -70ºC for further analysis.

2.5 REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

Real time PCR is used in the amplification of template DNA into multiple copies of (thousands or even millions) using a thermostable polymerase. The polymerase allows the extension of short single-stranded synthetic oligonucleotides (primers) during repeated cycles (20-40) of denaturation, primer annealing and primer extension in which the thermostable polymerase incorporates synthetic deoxyribonucleotides to duplicate the segments between the primers. These steps are temperature dependent and are carried out in the thermocycler. PCR is usually exponential initially and then plateaus (at different rates) when a reagent is limiting. At the exponential phase, the target amount doubles at every cycle (under normal condition) and the C_T (cycle number) value is taken, corresponding to when the signal is above the background. The resulting C_T are eventually resolved to determine the relative gene expression.

2.5.1 RNA Purification and measurement

Cells were seeded in 6 well-plate and at about 80-90% confluence, total cell lysate was made by first washing the cells 3X with PBS and then lysing the cells with lysis buffer containing 350µl buffer RLT (Qiagen®) and 6µl 3M DTT. The lysates were then scraped into eppendorf tubes and stored at -70ºC (if not using immediately).

Total RNA was purified from the cell lysates using the Qiagen RNeasy Mini kit, following the protocol as follows:

1. The lysates were pipetted into the QIAshredder spin column on a 2ml tube and centrifuged for 2 mins at maximum speed.

2. The homogenized lysates were transferred to a gDNA Eliminator column placed on new 2ml collection tubes followed by 30 second centrifugation at 10,000 rpm

3. The column from above was discarded and to the flow-through was added 350µl of 70% ethanol followed by gentle pipetting to mix

4. 700 μl of the above mix was then transferred to RNeasy spin column on 2ml collection tube and centrifuged for 30 seconds at 10,000 rpm. The flow-through was discarded while the collection tube was retained.

5. 700μl of buffer RW1 was added to the column and then centrifuged for 30 seconds at 10,000 rpm.

6. The flow-through from above was discarded while the column was reused. 500μl of buffer RPE was added to the column followed by 2 minutes centrifugation at 10,000 rpm

7. The tube with the flow-through was discarded while the column was placed on a new 2ml collection tube and centrifuged for 1 minute at maximum speed.

8. The RNeasy column was then placed on a new collection tube after which 40 μl of RNase–free water was added to the column, followed by a 1 minute centrifugation at 10,000 rpm to elute the RNA and kept on ice.

9. The concentration of the purified RNA was measured on the Nanodrop after which the samples were stored at -70℃.

2.5.2 The Nanodrop Technique

The Nanodrop technique involves the measurement of nucleic acid concentration in microliter volumes, using the Nanodrop spectrophotometer. It is a fast and easy method that simply requires the placement of sample directly on top of the detection surface and using surface tension to create a column between the ends of the optical fibres and forming the optical path. Its operational spectral range is between 220-770 nm with a sensitivity range of between 2 and 3700 ng/μl; and the total time utilized per sample measure is approximately 10 seconds.

Protocol:

Log in to the computer connected to the Nanodrop machine

Open the ND-1000 program

Select Nucleic acid – OK

Clean the Nanodrop sensor twice with 2 µl RNase-free water

Select RNA-40

Pipette 2 µl RNase-free water and click BLANK

Clean off the water with lens paper

Pipette 2 µl sample

Click on measure

Click on PRINT SCREEN

Clean with lens paper in-between samples

Click on PRINT REPORT

When done, clean off the last sample with lens paper, clean the sensor with 2 µl RNase-free water and secure the sensor with clean lens paper.

2.5.3 cDNA synthesis
cDNA synthesis is used to reverse transcribe template RNA into DNA copies.

The purified RNA sample was prepared for cDNA synthesis by standardizing it to 12ng/µl (according to the protocol). The sample was then made up to 12 µl by adding the appropriate volume of RNA-free water and kept on ice.

Master-mix 1 was prepared using 2µl per sample of gDNA wipeout and 2µl per sample RNase-free water. 4 µl of the mix was added to PCR tubes while 10 µl of the diluted RNA sample was added to the tubes on the RNA bench, in order to prevent RNA contamination. The first PCR was run on the GeneAmp PCR System 9700 (Life Technologies) at:

42°C for 2 minutes

4°C for 2 minutes

4°C for ∞

While the first PCR was running, Master-mix 2 was prepared using 1µl per sample RTenzyme, 4 µl per sample buffer and 1 µl per sample primer mix. 6 µl of the mix was then added to the PCR 1 product and PCR 2 was run at:

42°C for 15 minutes

95°C for 3 minutes

4°C for 7 minutes

4°C at ∞

The cDNA derived was measured on the Nanodrop and stored at -20ºC.

2.5.4 Polymerase Chain Reaction (SybrGreen Assay)

Sybr® Green is a double-stranded DNA intercalating dye that emits fluorescence when bound to DNA. It is the most commonly used dye for non-specific detection, requiring a pair of specific primers to amplify the target. The dye emits at 520 nm and the emitted fluorescence is detected and related to the amount of the target.

Master Mix 1 for the PCR was made with 12.5 µl Sybr green and 10.5 µl per sample

Master Mix 2 was also made for each of the primers for the PCR reaction (eg GAPDH, β-actin, MMP-2, MMP-9 etc), using 1 µl primer per sample + 23 µl Master Mix.

 24 µl of the Master-Mix 2 was pipetted into the corresponding test wells, to which 1µl of the respective cDNAs were added.

The no-template control contains only water and the reaction mix. It is used for the detection of contaminations, non-specific amplifications or primer dimers. Here, 1µl

RNase-free water was added to the wells containing Master-Mix 2 (SybrGreen, water and the respective primers).

The PCR was run on the Stratagene® MX3000 according to the protocol, using ROX as the dye.

2.6 WESTERN BLOTTING

Western blotting is a protein detection and analysis technique used for the separation of proteins according to their sizes, based on the ability of the proteins to bind to specific antibodies.

2.6.1 Sample Preparation (Cell lysates)

Approximately $0.3 - 0.6 \times 10^6$ cells were grown to confluency after which they were washed three times with PBS and then harvested with 100µl lysis buffer made up of 50 µl 4X LDS sample buffer, 30 µl milliQ water and 20 µl 0.1M DTT.

The cell lysates were stored at -20ºC when not using immediately.

For the western blot, the cell lysates were first sonicated at 15 cycles: 15 seconds ON and 15 seconds OFF, using the Bioruptor® plus diagenode machine. Samples were sonicated to disrupt their membranes and release their contents after which 30 µl samples were transferred to new eppendorf tubes after which and heated for 3 minutes at 85ºC. The samples were then loaded onto the gel (nuPAGE 1.5mm 4-12% Bis- Tris gel) and the nuPAGE gel electrophoresis was run for 35 minutes in 1x NuPAGE Running buffer (MES SDS) using the Invitrogen Lite technologies Power Ease 500 machine.

2.6.2 Blotting

After the gel run, blotting was done by first preparing the PVDF membrane (7.5cm by 8.5cm), which is the same size orientation as the gel. The PVDF membrane was soaked

for 3 seconds in methanol, 10 seconds in water and >5 minutes in blotting buffer. Two filters were also soaked in the blotting buffer. When the gels were done running, the cassette was broken using a gel knife and with the gel on the larger plate, the wells were removed. The pre-wetted filter was placed on the gel and turned (filter-down) on the work-bench after which the lowest part of the gel was removed. Pre-treated PVDF membrane was then placed on the gel, followed by the second pre-wetted filter. Air-bubbles were then removed by rolling out with a glass rod. Three pre-wetted pads were placed in the blotting module, followed by the "gel-in-PVDF and filter" set-up and finally the last three pre-wetted pads. The blotting module was placed in the cathode core after which the inner chamber was filled with blotting buffer and the outer chamber, with water for cooling. The Blot was run for protein transfer according to the protocol).

2.6.3 Detection

Following the blotting, the membrane was washed for 5minutes in 5ml TBST, blocked for 1 hour at room temperature (with rotation) and incubated overnight (with rotation) at 4ºC in primary antibody diluted appropriately in blocking buffer. The membrane was then washed with 5ml TBST for 3x5 minutes to remove the primary antibody and then incubated for 1 hour at room temperature with secondary antibody diluted appropriately in blocking buffer. After the secondary antibody incubation, the membrane was washed 3x 5 minutes in 5ml TBST, incubated for 1 minute in 1.5ml Luminol reagent using 1:1 ratio of solution A to solution B. The membrane was then sealed in a plastic wrap and exposed using the LEICA-3000® machine.

For Western blotting on cell supernatant, after 24 hours incubation, the medium from the cells was replaced with serum-free medium, after washing first with PBS. The cells

were incubated for 48hours, after which the medium was harvested and centrifuged for 10 minutes at 1600 rpm. Prior to storage at -70ºC, 10% freezing solution (1M Hepes solution pH7.4 and 0.1M CaCl$_2$) was added to the cell supernatant. The western blotting procedure for the conditioned medium was exactly the same as that of the cell lysates except for the omission of the sonication step, which is not necessary for cell supernatant.

2.7 GELATIN ZYMOGRAPHY

Gelatin Zymography is a method used in the detection of proteolytic enzymes that are able to degrade gelatin from various biological sources such as cell culture, cell conditioned medium, bodily fluids and tissue samples.

2.7.1 Conditioned Medium

Conditioned medium for zymography was generated in exactly the same way as that of the Western blotting.

2.7.2 SDS-PAGE

The samples were analysed by gelatin zymography in 7.5% separating gelatin gel and 4% stacking gel.

The separating gel was cast and left to polymerise for approximately 20 minutes after which it was topped with the stacking gel. The comb was inserted at this point and following polymerisation, the samples (7.5μl +1X loading buffer) were loaded onto the gel and run using the Pharmacia Biotech Electrophresis Power Supply (EPS 600) at a constant current of 18A (for 1 gel) and 36A (for 2 gels) for approximately 2 hours, in 1X electrophoresis buffer .

The gel was then washed for 2x30 minutes in washing buffer after which it was incubated overnight in incubation buffer

Following the overnight incubation, the gel was stained for 1 hour in 50ml staining solution and then destained in 50ml destaining solution for 1 hour, after which it was viewed under white light.

Gel photography was done using the UVP BioDocit ™ machine at 0-0-80 intensities

2.8 BACTERIAL TRANSFORMATION AND PLASMID PURIFICATION

Bacterial transformation is one the methods by which plasmids are introduced in competent bacterial cell, which in turn enables the amplification of the plasmid into multiple copies.

The plasmids used for the project were expanded by the transformation of the _E.coli_ DH5α competent bacteria. The plasmids were purified using the Qiagen Miniprep kit and the Macherey Nagel Nucleobond® Xtra Midi plasmid purification kit respectively.

The transformation of the _E. coli_ DH5-α competent bacteria was done by gently thawing 50-100µl of the bacteria on ice after which approximately 1-2µl of the plasmid was added to the bacteria, tapped gently to mix and incubated on ice for 30 minutes. Following the incubation, the bacteria was heat-shocked by incubating for 42 seconds at 42ºC and then returned to ice for 2 minutes. 300µl of pre-warmed SOC medium was added to the bacteria, which was then incubated with shaking at 37ºC and 182rpm for 16-18 hours.

2.8.1 Miniprep plasmid purification:

Following bacterial transformation, one colony of the transformant was inoculated into 5ml of prewarmed LB medium (100µg/ml ampicillin) and incubated overnight with

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shaking at 37ºC. Approximately 1ml freeze stock of the plasmid was made by adding 20% glycerol and freezing at -70ºC.

The rest of the bacterial culture was then purified using the Qiagen miniprep plasmid purification kit as follows:

The culture was centrifuged at 8000 rpm, the supernatant was discarded while the pellets were resuspended in 250μl of Buffer P1.The tube was tapped gently to mix and the resuspended cells were transferred to new eppendorf tubes

250μl of buffer P2 was added, followed by the inversion of the tube 4-6 times to achieve proper mixing after which the mix was incubated for 5 minutes at room temperature.

350μl of Buffer N3 was added to the mix followed by gentle mixing (inversion of the tube 4-6 times) and centrifugation at 13,000rpm for 10 minutes.

The supernatant was then applied to the spin column and centrifuged at 3,000rpm for 60 seconds after which the flow-through was discarded

The column was washed with 750μl of Buffer PE and centrifuged for 60 seconds.

The column was left to dry at room temperature for 10 minutes after which the collection tube was replaced.

50μl of the elution buffer was then added to the column, left to stand for 1 minute and then followed by 1 minute centrifugation at 12,000rpm to elute the DNA.

The concentration of the purified plasmid DNA was measured using the Nanodrop and the plasmid was stored at -20ºC.

2.8.2 Midiprep Plasmid purification
Following bacterial transformation, one colony of the transformant was inoculated in 100ml of prewarmed LB medium (100μg/ml ampicillin) and incubated overnight with

shaking at 37ºC. Approximately 1ml freeze stock of the plasmid was made by adding 20% glycerol and freezing at -70ºC.

The rest of the culture was centrifuged at 5000 x g for 10 minutes, at 4°C after which the supernatant was discarded and the plasmid purified using the Nucleobond® plasmid purification kit according to the protocol below:

1. The pellet was resuspended in 8ml resuspension buffer RES (plus added RNase). 8ml of the lysis buffer, LYS was added to the suspension and mixing was done by inverting the tube 5 times, followed by a 5 minute room temperature incubation to lyse the cells.

2. During the incubation period, the nucleobond® column as well as the inserted column, was equilibrated by applying 12ml of the equilibration buffer EQU around the rim of the filter.

3. Following the 5 minute incubation period, 8ml of the neutralization buffer, NEU was then added to the cell lysate which was gently mixed by inverting the tube 15 times.

4. The lysate was then gradually clarified (by inverting the tube 3 times) and loaded onto the column in the same manner as the equilibration buffer EQU was applied (around the rim of the filter).

5. The column filter was then washed with 5ml equilibration buffer after which the filter was discarded and the column was washed with 8ml wash buffer, WASH.

6. Following the washing, the plasmid DNA was eluted by directly adding 5ml elution buffer, ELU.

7. 3.5ml room-temperature isopropanol was then used to precipitate the eluted plasmid DNA after which it was vortexed properly and incubated for 2 minutes at room temperature, followed by centrifugation at 12,500 x g for 30 minutes at 4°C.

8. The supernatant was carefully discarded while the pellet was washed with 2ml 70% ethanol and centrifuged at 12,500 rpm at room temperature for 5 minutes.

9. Following the last centrifugation, the supernatant was discarded carefully and the pellets were dried at room temperature for 15 minutes after which the DNA pellet was dissolved in 500µl TE buffer. The DNA concentration was measured on the Nanodrop after which the DNA solution was stored at -20°C for later usage.

2.9 RESTRICTION ENZYME DIGESTION AND AGAROSE GEL ELECTROPHORESIS

Restriction enzyme digestion is the use of restriction endonucleases to break the sugar-phosphate backbone of bacterial DNA at specific nucleotide sequences known as the restriction sites, in order to generate smaller fragments.

Agarose gel electrophoresis is used in the analysis of DNA fragments that have been generated by restriction enzyme digestion, according to their molecular sizes which range from between 500 to 30,000 base pairs.

Plasmid Digestion:

The restriction enzyme digestion was performed by combining the following in a 1.5ml reaction tube:

1µg plasmid

1µl restriction enzyme (Nde1 R0111S Lot 0280805)

2.8µl 10X Buffer (NEB Buffer 2 B 70025 Lot 1205 A)

Distilled water (for a total volume of 25 μl of the reaction mix)

Overnight Incubation at 37°C

2.9.1 Gel preparation:

1% agarose gel was prepared by mixing 1g agarose gel powder in 100ml 1X TAE buffer,

which was dissolved by heating in the microwave. The gel was poured up to about 0.5cm

in a gel plate and left to polymerise for bout 20-25 minutes after comb insertion.

Both the digested and undigested samples were prepared with 6X loading dye (

Fermentas® #R0611 Lot 00000802)

The "set" gel was transferred to the electrophoresis chamber after which 1XTAEbuffer

was used to cover the gel. The samples, both digested (for test) and undigested (for

control) as well as the 100bp (Fermentas GeneRuler™ 100bp DNA Ladder plus #SM 0321

Lot 00016171) and 1kb molecular weight standards (Fermentas Ladder Gene Ruler™

1kb DNA ladder plus #SM1331 l Lot 00000201) were loaded on the gel in the correct

order. The gel was run for 1hour at a constant voltage of 90V.

2.9.2 Staining and Development

The gel was stained in 20% staining solution (Biotrun Gel Red Nucleic acid stain Lot

11G0810) for 30 minutes with slight shaking after which it was washed slightly in water

and then developed using the BioRad GelDoc 2000 machine.

2.10 DNA SEQUENCING

DNA sequencing is a process used in the determination of the order of nucleotide sequences along a DNA strand.

The sequencing reaction was carried out by making mixes of the following, for the forward and reverse primers respectively:

1-3 ng 5X Sequencing buffer

3-10 ng Big Dye 3.1

5-20 ng DNA template

10-40 ng forward and reverse primers respectively

The mix was made up to 20µl with nuclease-free water

The sequencing PCR was run using the program:

 30 cycles

96°C - 10 secs

50°C - 10 secs

60°C - minutes

4°C - infinity

The sequencing PCR product was eventually delivered to the sequencing platform for further analysis after which a nucleotide BLAST search was done on PUBMED.

2.11 IMMUNOSTAINING AND CONFOCAL MICROSCOPY

Immunostaining and confocal microscopy is used in the detection of an antigen in cell or tissue sections by means of antigen-antibody recognition. Confocal microscopy is employed to determine the subcellular localization of the antigen.

10,000 cells were seeded in the 8-well chambered glass slide up to a total medium volume of 200µl and incubated overnight at 37°C.

After the overnight incubation, the medium was aspirated after which the cells were fixed using 8% ice-cold PFA in PBS and incubated on ice for 10 minutes.

The cells were then washed twice with 1X PBS and permeabilized by adding 200µl 0.1% Triton x100, followed by a 5 minute incubation at room temperature.

Blocking was done by first washing the fixed cells twice with 1X PBS and incubating in 3% goat serum in PBS for 30 minutes at room temperature after which the blocking solution was aspirated and the cells incubated in 200 µl primary antibody in 1% goat serum in 1X PBS for 1 hour at room temperature.

The primary antibody was aspirated after the 1 hour incubation and the cells were washed 6 times with 1X PBS after which they were further incubated with 200µl 1:200 secondary antibody in 1% goat serum in 1X PBS for 30 minutes at room temperature.

The cells were then finally washed 6 times with 1XPBS and analysed using confocal microscopy.

2.12 IN SITU ZYMOGRAPHY

In situ zymography is a technique that aids in the detection of enzymatic activity in cell culture or tissue sections. Detection by this method occurs when a substrate is deposited and incubated on or under cells or tissue and the nature of the substrate used will determine the actual enzyme that will be detected.

Gel preparation:

2% sucrose and 2% gelatin (porcine) gel was prepared by weighing 0.2g gelatin and 0.2g sucrose, to which were added 1x sterile PBS up to 10 ml. The mixture was heated in 56-62°C water bath till the solution became homogenized after which it was sterile-filtered using a 0.2μm filter and then left to cool down slightly.

The DQ gelatin was prepared by adding 1ml sterile water to 1mg DQ-gelatin powder and mixed properly.

A 1ml working solution containing 25μg/ml DQ-gelatin was then prepared using 975μl filtered 2% gelatin and 2% sucrose solution + 25μl DQ-gelatin (1mg/ml) out of which 100μl was pipetted into each of the chambered glass slide wells.

The chambered glass slide was then incubated for 10 minutes at 4°C and following polymerization, about 100,000cells in 100μl serum-free medium was added per well.

The glass slide was incubated for about 10 minutes after which the cells were visualized under the confocal microscope for gelatinolytic activity which is seen as green fluorescence.

2.13 xCELLigence System

The xCELLigence system uses the principle of microelectronic cellular impedance technology to monitor the behaviour of cells such as adhesion, proliferation, migration and cytotoxicity in real time.

2.13.1 Cell adherence and proliferation

For the adherence and proliferation assay, 50μl of the complete growth medium was pipetted into each of the wells of the E-16 plate after which the plate was equilibrated by incubating for 1 hour at 37°C.

Following the 1 hour incubation, a background measurement was done using the step 1 of the xCELLigence RTCA SW.

The cells were then washed, trypsinized and counted, out of which the required amount of cells in a total of 100µl was added per well. The plate was incubated under the hood for 15-20 minutes after which it was placed in the DP system for measurement at 5 minutes interval for 12 hours and 15 minutes interval for 72 hours.

2.13.2 Cell migration

160µl of complete growth medium was added to each of the BC (bottom chamber) wells of the CIM-16 plate after which the BC and the TC (top chamber) were assembled in the proper orientation. 30µl of SFM was then added to the TC wells after which the assembled CIM plate (with medium) was incubated for 1 hour at 37°C followed by background measurement using step 1 of the xCELLigence RTCA SW.

The cells were washed with PBS and trypsinized with trypsin-EDTA. Trypsinization was stopped with a few milliliters of media containing serum after which they were centrifuged at approximately 800g for 5 minutes.The pellets were washed by resuspension in SFM and centrifuging a second time for 5 minutes at 800g. Following the washing, the cells were again resuspended in SFM after which they were counted and 100µl of the cell suspension containing the appropriate cell number added to each of the TC wells of the CIM plate.

The plate was incubated under the hood for 15-20 minutes after which it was placed in the DP system for measurement at 15 minutes interval for 72-84 hours.

2.13.3 Cell invasion

Matrigel was defrosted on ice and diluted (1:3) in SFM for a total of 800μl out of which 50μl was added to each of the TC wells of the CIM-16 plate (sitting on the BC for support) using cool pipette tips always. 30μl of the matrigel was taken out, leaving the remaining 20μl to coat the wells. The plate was incubated for 10 minutes at 37°C after which 160μl medium with FBS was added to the BC wells. The TC and BC were then assembled in the proper orientation followed by the addition of 25μl SFM to cover the matrigel in the TC, after which the plate was incubated at 37°C for 1 hour. The background was measured using step 1 of the xCELLigence RTCA SW after which cells in 100μl SFM were added to the TC wells. The CIM plate with cells was incubated in the hood for 15-20 minutes, to allow the cells settle, after which it was placed in the DP system for measurement at 15 minutes interval for 72-108 hours.

3. RESULTS

The results that are presented in this thesis have been divided into 3 main sections:

MMP-2 overexpression, MMP-14 knockdown, and the analysis of the effects of the

overexpression and the knockdown on the cells.

3.1 Determination of MMP-2 expression levels in wild-type HSC-3 cells

Going by the hypothesis that MMP-2 could also have some intracellular

function, an overexpression of the intracellular isoform of the protein was important to

help investigate these functions especially in terms of cell migration and invasion. Using

the HSC-3 cells, there was the need to ensure that they were not endogenously

expressing the protein and thus Western blotting was employed to ascertain the

expression level of the MMP-2 protein in HSC-3 cell lysates (Fig. 7)
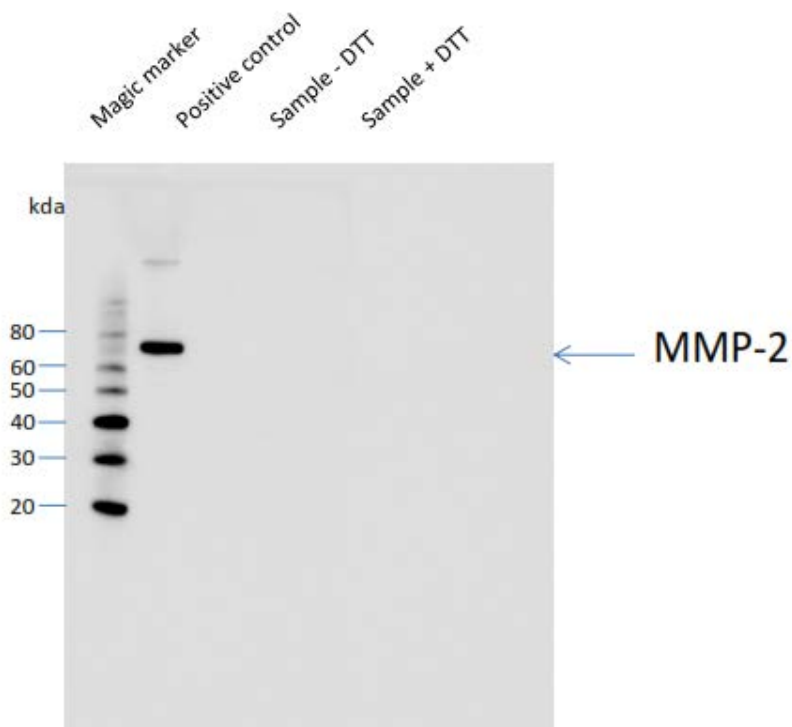
Figure 7. HSC-3 cells do not endogenously express MMP-2 protein.
Western Blot analysis for MMP-2 expression on HSC-3 wild-type cell lysates was performed by seeding 1.0×10^6 cells of the cells which was harvested at confluence using 100μl lysis buffer and run on 4-12% bis-tris gel.
Lane 1: molecular weight standard (Magic Marker); lane 2: positive control (conditioned fibroblast medium; lane 3: 30 μl HSC-3 cell lysate (-DTT); lane 4: 30 μl HSC-3 cell lysate (+DTT).The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and detected by ECL using the Goat anti-human-MMP-2 primary antibody (AF902 R&D) (1:500) and the HRP-conjugated anti-goat/sheep secondary antibody (A9452 Sigma-Aldrich) (1:100,000).

As shown in figure 7, MMP-2 protein with the expected molecular weight of 72kDa was identified in the positive control sample (lane 2), while no MMP-2 could be detected in the HSC-3 samples (lanes 3 and 4). As a result of the finding, the cells were deemed very suitable for the proposed protein overexpression studies with the MMP-2 plasmid.

3.2 MMP-2ic overexpression in HSC-3 cells

Since HSC-3 cells do not express any endogenous the MMP-2 protein, they were transfected with the plasmid, Ex-Z5731-m02-MMP-2 (GeneCopoeia), which contain an insert of transcript variant 2 of MMP-2. This variant encodes a protein which lacks the first 54 amino acids of MMP-2 (named MMP-2ic), hence it lacks the signal peptide and parts of the prodomain and is therefore cytoplasmic and possibly active. Another plasmid, Ex-neg-M02 (GeneCopoeia), lacking the MMP-2 insert served as an empty vector control for the overexpression. Prior to the overexpression, the plasmids were transformed into the *E.coli* DH5-α bacteria for expansion, followed by purification with the Qiagen® Miniprep plasmid purification kit (and subsequently with the Nucleobond® Xtra Midiprep). In order to confirm the identity, the purified plasmids were subjected to restriction enzyme digestion and agarose gel electrophoresis (Figure 8).

Figure 8: Nde 1 restriction enzyme digestion of Ex-Z5731-M02-MMP-2 and Ex-neg-M02 Plasmids.
Agarose gel electrophoresis for the verification of cDNA encoding intracellular MMP-2 ((Ex-Z5731-M02-MMP-2) and Empty vector (Ex-neg-M02). 6.3μg of the purified plasmids were digested with 20U of the Nde1 restriction enzyme. The samples were prepared by adding 6X loading buffer (Fermentas) to 10μl of the sample, after which they were run on 1% agarose gel.
Lane 1: Undigested MMP-2 plasmid (U1); lane 2: digested MMP-2 plasmid (D1); lane 3: Undigested empty vector plasmid (U2), land 4: digested empty vector plasmid (D2); lane 5: 1 kb molecular weight standard (NEB); lane 6: 100bp DNA ladder (Fermentas GeneRuler™). Gel staining was done with GelRed nucleic acid stain (Biotium) followed by imaging on the BioRad Gel Doc™ 2000.

Figure 8 shows the presence of one main band of about 7656bp for the undigested

MMP-2 plasmid (lane 1), two bands for the digested MMP-2 plasmid with sizes 2590bp

and 5066bp (lane 2); one main band for undigested empty vector plasmid with a size of

5798bp (lane 3) and 2 bands for the digested empty vector plasmid with sizes 732bp

and 5066bp (lane 4) on 1% agarose gel. The detected bands for the digested plasmids

corresponded to the calculated fragment sizes that would be generated by the Nde1 restriction enzyme, as deduced from the plasmid map.

The above result notwithstanding, the purified MMP-2 plasmid DNA was sequenced for further confirmation of the plasmid identity. DNA sequencing was done on two individually purified MMP-2 plasmids using both forward (5'- CAGCCTCCGGACTCTAGC-3') and reverse (5'- TAATACGACTCACTATAGGG-3') primers for the forward and backward reactions respectively (Appendix: figure 1).

Figure 1 in the appendix shows the nucleic acid sequences generated from the purified plasmid. A nucleic acid BLAST search on NCBI revealed about 98% complementarity between the sequences of the MMP-2ic insert and the human MMP-2 transcript variant 2 (Accession number NM 001127891) found in the database.

3.3 Confirmation of Intracellular MMP-2 (MMP-2ic) overexpression in HSC-3 cells

Single cell clones generated by the overexpression of the intracellular MMP-2, were provided for this work (12 HSC-3-MMP-2ic clones and 2 HSC-3-EV clones), and in order to proceed with the proposed study, the clones were analysed for the overexpression of the protein using gelatin zymography.
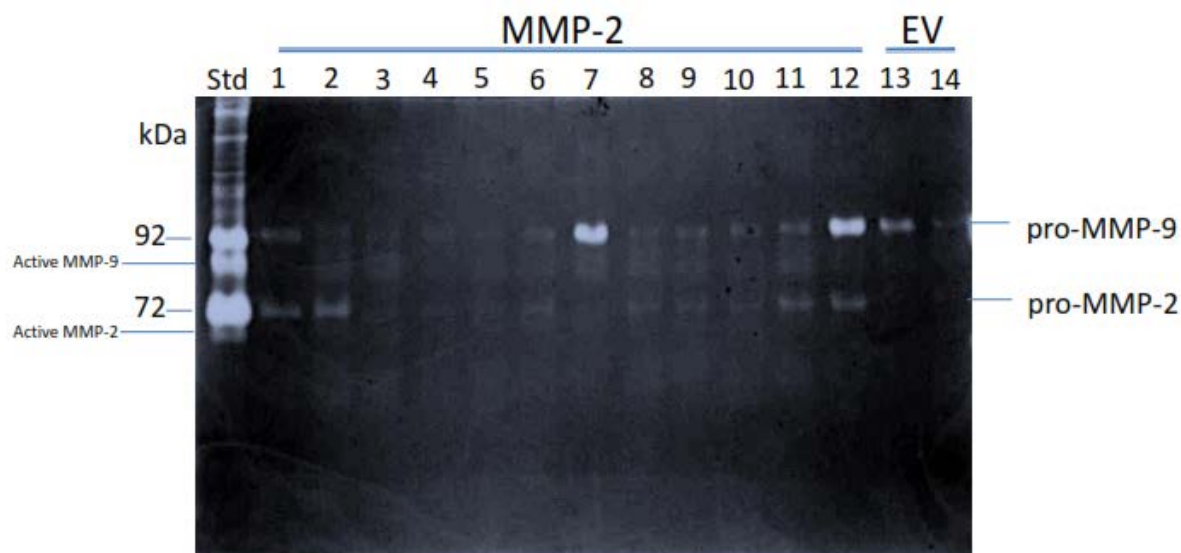
Figure 9: Conditioned cell medium from HSC-3 cells overexpressing MMP-2ic degraded gelatin. Conditioned media from 12 stably selected HSC-3-MMP-2ic clones and 2 for empty vector clones were prepared with 5X loading buffer and run on the SDS-PAGE for enzymatic activity.
Lane 1: Positive control for human pro-MMP-9, active MMP-9, pro-MMP-2 and active MMP-2; lanes 2-13: 7.5µl HSC-3 conditioned medium for the HSC-3-MMP-2ic clones respectively; lanes 14 & 15: 7.5µl HSC-3 conditioned medium for the empty vector clones respectively. Gel staining was done with Coomassie blue containing 20% acetic acid and gelatinolytic activity was seen as clear bands on a blue background.

Figure 9 shows bands with an apparent size similar to pro-MMP-2 with varying degrees of gelatinolytic activity (lanes 2-13). No bands with the size corresponding to MMP-2 were observed for the empty vector clones (lanes 14 and 15). In all samples, a band corresponding to pro-MMP-9 could be observed on the zymogram. That notwithstanding, there were no observed bands for either active MMP-2 or MMP-9 on any of the samples.

As a further confirmatory test for MMP-2ic overexpression, an RT-qPCR was run on total RNA from the cell clones. RNA was extracted from the cell lysates using the Qiagen RNeasy kit. Purified RNA of ≥ 100ng/µl were sent out to the integrity testing platform, to ensure that they were suitable for further analysis (Figure 10A).
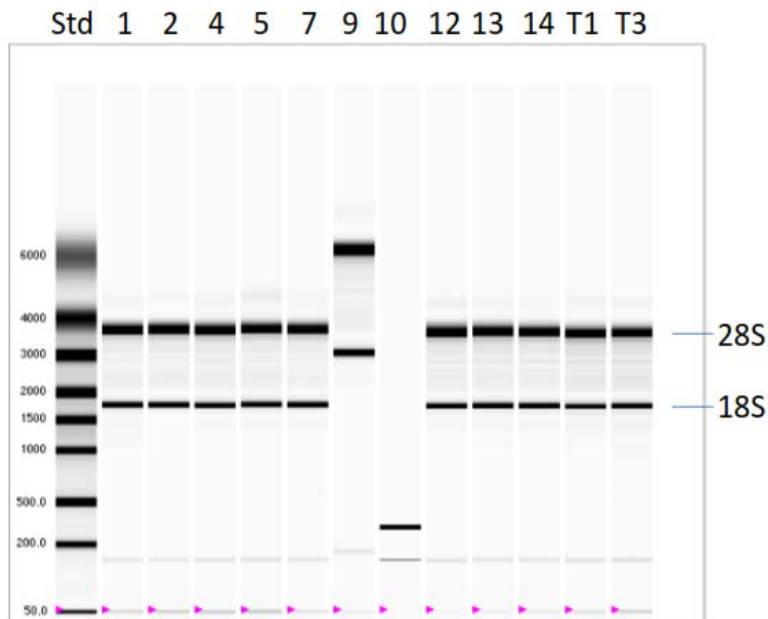
Figure 10: Intact RNA were isolated from most of the HSC-3-MMP-2ic and HSC-3-EV single cell clones

Gel electrophoresis using the Bioanalyzer showing the integrity of RNA after purification. The RNAs with measured concentrations of >100ng/µl were tested for integrity at the platform using the Experion RNA StdSens.

Lane 1: Molecular weight standard (Experion StdSens); lanes 2-9: RNA from stable MMP-2 clones 1, 2, 4, 5, 7, 9, 10 and 12; lanes 10 and 11: RNA from stable empty vector clones; lanes 12 and 13: RNA from transient transfection with PmaxGFP and MMP-2ic respectively.

Figure 10 shows that integrity testing yielded intact RNA bands corresponding to the 28S and 18S ribosomal RNA (lanes 2-6 and 9-13) as well as some degraded RNA (lanes 7 and 8). The samples with degraded RNA were excluded from further analysis by RT-qPCR.

An RT-qPCR was run on the intact RNA samples using SybrGreen assay, with *Gapdh* and *β-actin* serving as the reference genes. The C_T values derived from the RT-qPCR were normalized to the internal controls after which the fold change was determined by setting HSC-3-EV7 as 1 (Figure 11).
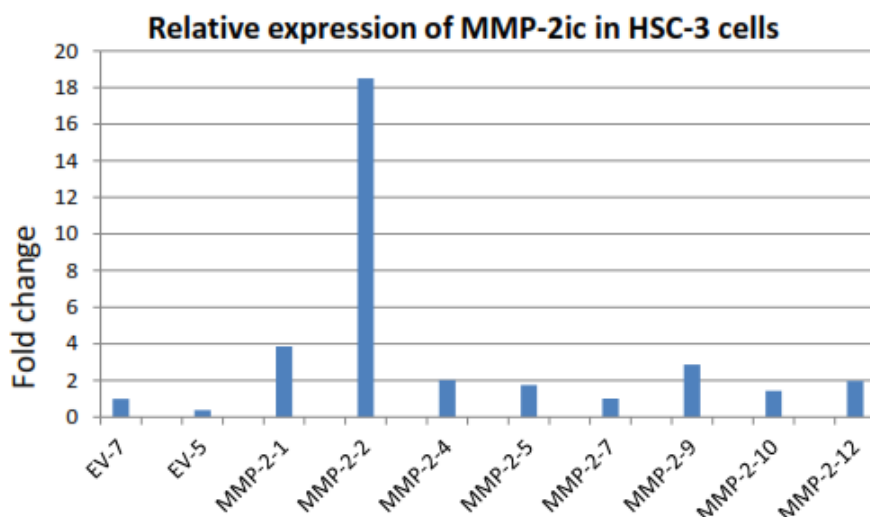
Figure 11: Stable transfection of HSC-3 cells with Ex-Z5731-M02-MMP-2 resulted in an increase in the *MMP*-2ic gene expression.
RT-qPCR result shows the relative expression of MMP-2ic in HSC-3 cell clones using SybrGreen dye assay. *Gapdh* and *β-actin* were used as the reference genes. Lanes 1&2: HSC-3-EV clones; lanes 3-10: HSC-3-MMP-2ic clones 1, 2, 4, 5, 7, 9, 10 and 12.

Figure 11 shows the increase in fold of MMP-2ic expression in HSC-3 cell clones relative to the empty vector control (EV-7), when the expression level of the empty vector was set at 1. The MMP-2ic mRNA expression levels were highest in HSC-3-MMP-2-2ic and lowest in HSC-3-MMP-2-7ic. The RT-qPCR results correlate with the results from the zymography, in which the strongest band was observed in HSC-3-MMP-2-2ic and the weakest in HSC-3-MMP-2-7ic.

3.4 MMP-14 Knockdown

In order to effectively determine the role of MMP-14 in HSC-3 cell migration and invasion, it was of paramount importance to determine first if the protein was endogenously expressed in HSC-3 cells. To ascertain this, Western blot analysis was carried out on the HSC-3 cell lysates (Figure 12).
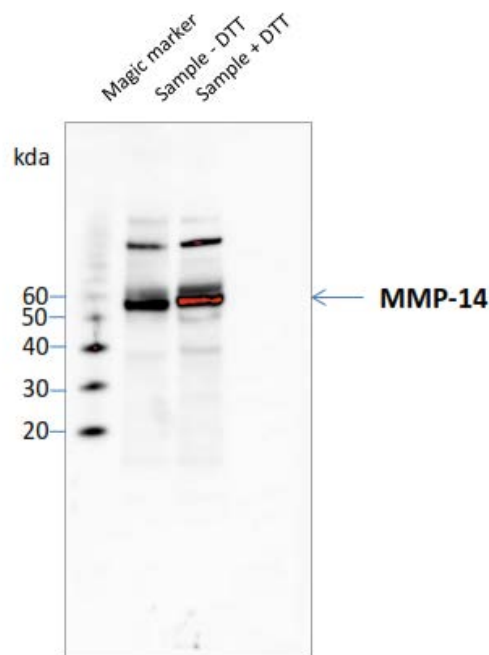
Figure 12: HSC-3 cells endogenously express MMP-14.
Western Blot analysis for MMP-14 expression in wild-type HSC-3 cell lysates was carried out by seeding 1 x 10^6 cells which were harvested at confluence with100µl lysis buffer and run on 4-12% Bis-Tris gel.
Lane 1: molecular weight standard (Magic Marker); lane 2: 30 µl HSC-3 cell lysate (-DTT); lane 3: 30 µl HSC-3 cell lysate (+DTT).
The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and detected by ECL using Rabbit anti-human MMP-14 primary antibody (Novus EP1264Y) (1:2000) and Goat anti-rabbit IgG H+L chain specific secondary antibody (Southern Biotech) (1:2000).

Figure 12 shows that MMP-14 protein with the expected molecular weight of approximately 66kDa was detected in the HSC-3 samples, both in the presence and absence of DTT.

3.4.1 Transient transfection using shRNAs for MMP-14

To study the role of MMP-14 protein in HSC-3 cell, an shRNA strategy was chosen for knockdown of MMP-14. In order to determine which shRNA construct that would give the most efficient knockdown, 4 different shRNA construcs were tested by transient transfection of the HSC-3 cells (Figure 13).
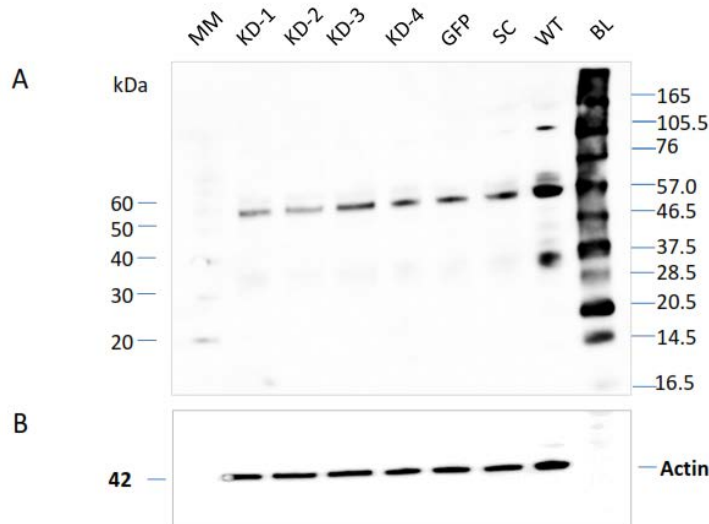
Figure 13A: MMP-14 protein expression was reduced by MMP-14 shRNAs.
Western blot analysis was carried out on HSC-3 cell lysates after 0.3×10^6 HSC-3 cells were transfected on a 6-well plate using 2.5µg shRNA plasmid DNA.
The cells were harvested 48 hours post-transfection using 300 µl RIPA buffer (+ protease inhibitor) after which equal concentrations (20 µg) of the sample was prepared with sample buffer and loaded on 4-12% bis-tris gel.
Lane 1: molecular weight standard (Magic Marker); lanes 2-5: lysates of shRNAs 1-4 transfectants respectively; lane 6: lysate of HSC-3 transfected with pmaxGFP; HSC-3 cell lysates (GFP); lane 7: lysate of HSC-3transfected with scrambled control; lane8: wild-type HSC-3 cell; lysates; lane 9: biotin ladder.
The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and detected by ECL using Rabbit anti-human MMP-14 primary antibody (Novus EP1264Y) (1:2000) and Goat anti-rabbit IgG H+L chain specific secondary antibody (Southern Biotech) (1:2000).
Figure 13B: As a loading control, the blot was reprobed with anti-β-actin (Sigma) (1:1000).

Figure 13 shows that MMP-14 shRNA constructs 1 and 2 gave the most knockdown in HSC-3 cells (lanes 2 and 3). The extra bands in the HSC-3 wild-type denotes some level of degradation of the protein (lane 9). Probing with anti-β-actin antibody revealed bands of approximately 42kDa, which correpond well with the expected size of human β-actin.

3.4.2 Stable transfection of shRNA for MMP-14

Based on the above result, the MMP-14 shRNA constructs 1 and 2 were further used to stably transfect HSC-3 cells for MMP-14 knockdown using puromycin as the stable selection marker. In this case, bulk transfections were done for the selection of mixed

clones. The knockdown was verified by Western blotting (Figure 14) and RT-qPCR

(Figure 15).
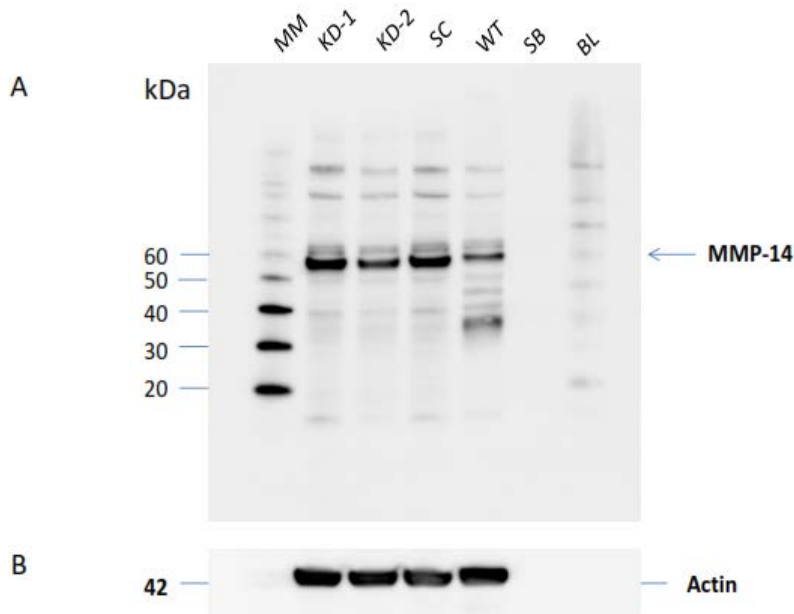
Figure 14A: Stable transfection of HSC-3 cells with shRNA1 and shRNA2 resulted in a detectable knockdown of MMP-14 in cells transfected with shRNA2 only

Bulk transfectants generated from the stable transfection of HSC-3 cells with shRNA constructs 1 and 2 (HSH011268-(1-2)-nU6) and a scrambled control (CSHCTR001-nu6) were analysed by loading equal amounts of the protein (+sample buffer) on 4-12% Bis-Tris gel.
Lane 1: molecular weight marker (Magic Marker); lanes 2: 30µl HSC-3 cell lysates (shRNA 1); lane 3: 30 µl HSC-3 cell lysate (shRNA 2); lane 4: HSC-3 cell lysates (scrambled control); lane 5: 30µl HSC-3 cell lysates (wild-type); lane 6: sample buffer; lane 7: biotin ladder.
The proteins were resolved by SDS-PAGE, transferred on nitrocellulose membrane and probed with Rabbit anti-human MMP-14 primary antibody (Novus EP1264Y) (1:2000) and Goat anti-rabbit secondary antibody (Southern Biotech)(1:2000).
Figure 14B:A reprobe for loading control was carried out using anti-β-actin (A3854 Sigma) (1:1000).

Figure 14A shows that only MMP-14 shRNA construct 2 was able to knockdown MMP-

14 expression in HSC-3 cells after stable transfection. This is seen in the fainter band

corresponding to approximately 66kDa, which is the molecular weight for MMP-14 in

lane 3. The extra bands in lane 5 is likely due to protein degradation in untransfected

HSC-3 cell lysate. Probing the blot with anti-β-actin antibody yielded 42kDa band sizes

of equal sizes equivalent to the molecular size of actin across all the samples (Figure

14B). This shows that equal amount of protein (for all the samples) was loaded on the gel, and also that shRNA 2 gave a detectable reduction in MMP-14 protein levels.

3.5 Analyzing MMP-14 knockdown in HSC-3 cells through RT-qPCR

To further verify knockdown in the transfected HSC-3 clones, the samples were analyzed by RT-qPCR. The RT-qPCR was run on the purified total RNA from both the transiently transfected cells and the bulk transfected clones using the SybrGreen assay and *Gapdh* as the internal control (Figure 15).
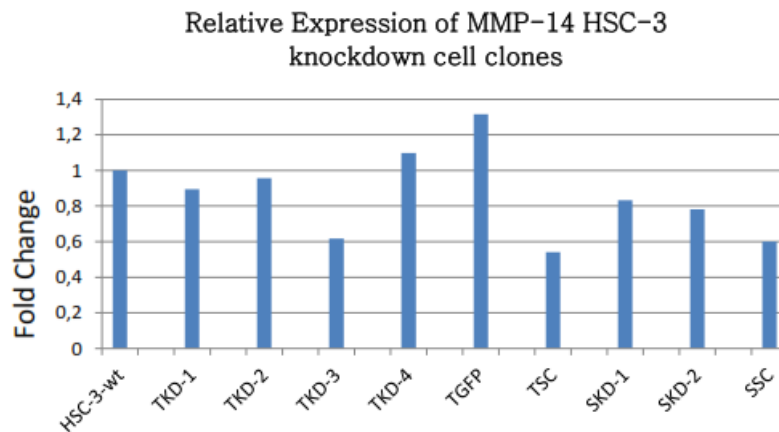
Figure 15: Stable transfection with MMP-14 shRNA can knockdown MMP-14 expression.
PCR run on MMP-14 clones was done with the Sybr Green dye using *Gapdh* as the internal control. The derived C_T values were normalized to the reference, and the fold change was calculated relative to the normalized HSC-3 wt expression level that was set to 1.
HSC-3wt; HSC-3 wild type,TKD-1-4; transiently transfected HSC-3 cells using shRNA constructs 1-4 respectively,TGFP; transiently transfected HSC-3 cell using pmaxGFP, TSC; HSC-3 cell transiently transfected with scrambled control, SKD-1-2; stably transfected HSC-3 cells using shRNA constructs 1-2 and SSC; stably transfected HSC-3 cells using scrambled control.

Figure 15 shows that MMP-14 expression was not reduced since the scrambled controls gave lower values than the MMP-14 shRNA constructs. Since the experiment was done once, it would have to be repeated to be more conclusive.

3.6 Analysing the cell features in terms of MMP-2 and MMP-14 localization, gelatinolytic activities, migration and invasion

Having overexpressed MMP-2ic and knocked down MMP-14, the effects were analysed by immunostaining (to check for the subcellular localization of the proteins and expression levels) and xCELLigence assay (for their migrative and invasive behavior). In situ zymography was carried out on a full-length version of MMP-2 (FL-MMP-2)* and empty vector to check for enzymatic activities.

*Even though the FL-MMP-2 plasmid was transformed and purified by me, the actual transfection was done by a colleague who provided me with these cells.

3.6.1 Immunostaining and confocal microscopy for the expression levels and subcellular localization of MMP-2 and MMP-14

Immunostaining was performed on all the cell clones by seeding 1×10^5 cells in 8-well chambered glass slides in which the cells were fixed (Figure 16).
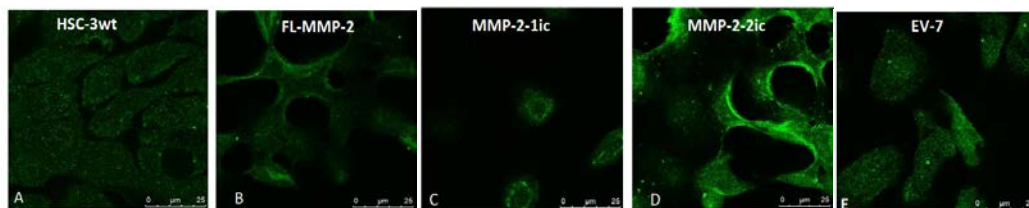
Figure 16 (A-E): Probing of MMP-2 overexpressing cell clones with specific antibodies revealed the subcellular localization of MMP-2 proteins.
The subcellular localization of the MMP-2 and MMP-14 proteins was determined by fixing the cells with 4% PFA.
Panel A: HSC-3 wt; Panel B: full length MMP-2;Panel C: Intracellular MMP-2-1; Panel D: Intracellular MMP-2-2; Panel E: Empty vector for MMP-2.
Probing was done with anti-MMP-2 primary antibody (Abcam ab37150) (1:100) and Alexa Fluor® 488 Goat anti-rabbit (Life Technologies® #A11034) secondary antibody(1:200).

Figure 16 (A-E) shows the presence of weak and diffuse MMP-2 staining in HSC-3wt (panel A). Increased fluorescence activities were observed around the cell edges of the MMP-2fl and MMP-2-2ic (panels B&D) compared to wild-typeHSC-3, MMP-2-2ic and the EV clones (panels A, C& E).
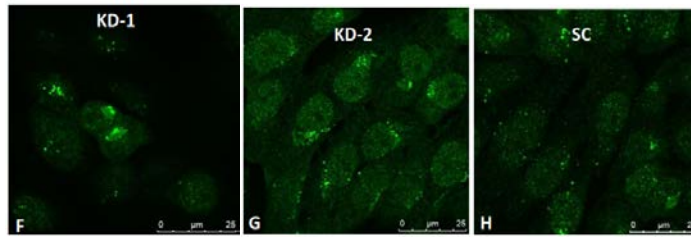
Figure 16 (F-H): Probing of MMP-14 knockdown cell clones with specific antibodies revealed their subcellular localization
The subcellular localization of the MMP-14 proteins was determined by fixing the cells with 4% PFA.
Panel F: MMP-14 knockdown clone 1; Panel G: MMP-14 knockdown clone 2; Panel H: MMP-14 scrambled control.
Probing was done with an anti-MMP-14 primary antibody (Novus NB 110-57216) (1:400) and Alexa Fluor® 488 Goat anti-rabbit (Life Technologies® #A11034) secondary antibody (1:200)
Confocal microscopy was used to analyze the cells for subcellular localization of the proteins which was observed as green fluorescence, using the 63x1.2 water objective lens.

MMP-14 KD clones showed almost the same distribution of fluorescent patterns (panels F-H), with what looked like an accumulation of punctuated fluorescence around the edges.

3.6.2 Investigation of gelatinolytic activity in FL-MMP-2 by in situ zymography
The FL-MMP-2 was further analyzed for gelatinolytic activity using EV as a control (Figure 17).
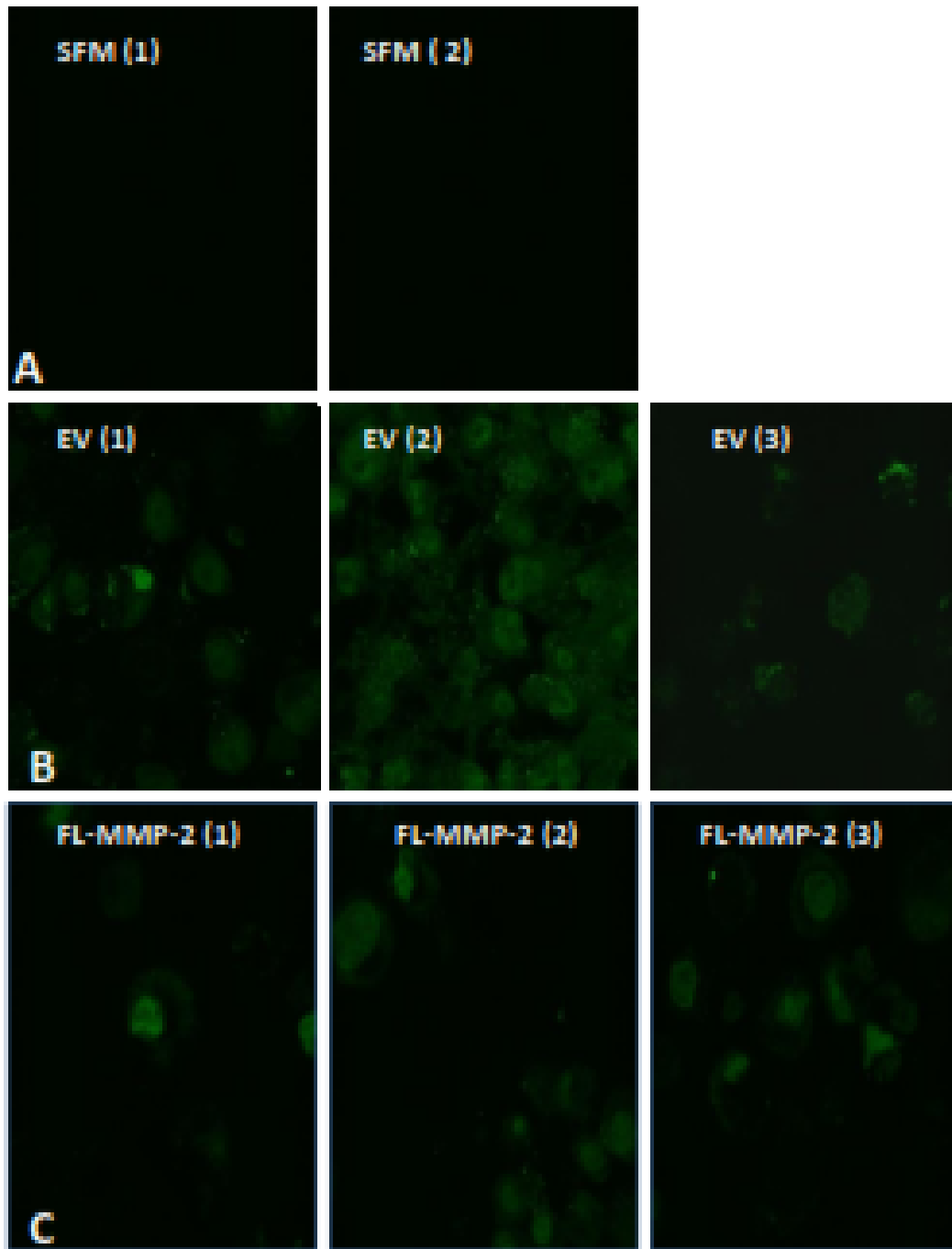
Figure 17: Gelatinolytic activity was demonstrated in HSC-3 selected for the overexpression of FL-MMP-2 and cells transfected with the empty vector plasmid.
In situ zymography was performed by seeding 1 x10^5 cells in an 8-well chambered glass slide coated with 100 µl 2% sucrose gel containing 2% DQ gelatin (Invitrogen D12054).
Top panel (A): Serum-free medium, wells 1& 2; Middle panel(B): Empty vector clones; Bottom panel (C): Full length MMP-2 clones.
The cells were incubated at 37°C after which they were analysed for gelatinolytic activity using 63x 1.2 water objective on the confocal microscope.

As shown in figure 17, the absence of green fluorescence indicates the absence of

gelatinolytic activity in the SFM, which contained no cells (Figure 17A). The empty

vector clone surprisingly displayed some gelatinolytic tendencies as indicated by the green cells (Figure 17 B) while the FL-MMP-2 clones showed anzymatic degradation of the DQ gelatin around the cells and in the nucleus (Figure 17 C).

3.7 Analysis of cell clones for proliferation, migration and invasion by xCELLigence assay

Having overexpressed the full-length version of MMP-2 (FL-MMP-2) and intracellular MMP-2 (MMP-2ic), as well as knock down MMP-14 in HSC-3 cells, the behavior of these cell clones in terms of migration and invasion was studied with the xCELLigence assay method.

3.7.1 Cell proliferation

At the start of the analysis, it was important to determine the optical number of cells that would maintain the exponential growth phase over a long period of time. In order to achieve this, different number of the HSC-3 cell (100, 300, 1000, 3000, 10,000, 30,000 and 100,000) were seeded in 100 μl complete growth medium in duplicates followed by analysis on the xCELLigence machine (Figure 18).
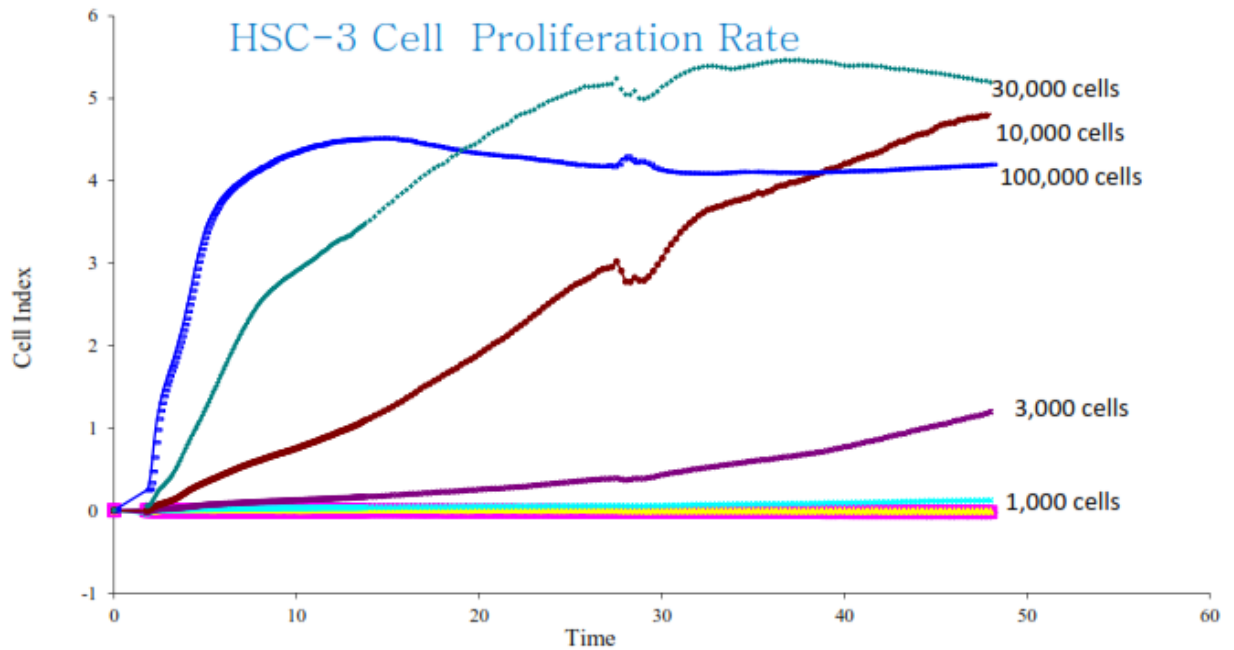
Figure 18: Optimal cell seeding density can be determined by the xCELLigence system.
Different number of HSC-3cells were seeded in duplicate on the the E-16 plate, in 100 μl of
complete growth medium and recorded for 50 hours. Impedance reading over time provided the
cell index which increased with increase in proliferation.

Figure 18 shows that the optimal seeding density that would maintain the HSC-3 cells in

a viable state was 10,000 cells. This cell number was used subsequently to analyse the

different cell clones for adherence and proliferation (Figure 19). Since all the different

cell clones were derived from the HSC-3wt cell, it was assumed that the same seeding

density derived from the wild-type cells would be applicable to the rest.

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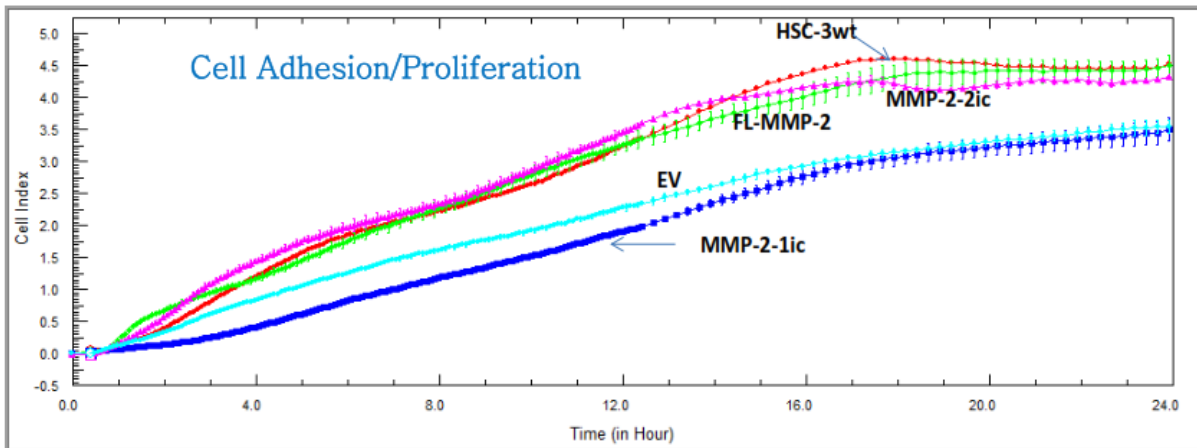
Figure 19: Adhesion and proliferation profile for MMP-2 clones.
10,000 cells in 100 µl of complete growth medium (for the 8 different cell clones) were seeded in duplicates on the E-16 microplate following the equilibration of the plate wells with 50 µl medium and background reading and recorded for 24 hours.
Plate well colour code: Red: HSC-3wt; Light green: FL-MMP-2; Blue: MMP-2-1ic; Pink: MMP-2-2ic; Light blue: Empty vector. The cells were monitored for 48-96 hours on the xCELLigence machine.

Figure 19 shows a similarity in the cell adhesion/proliferation pattern across the MMP-2 cell clones, with the exception of the EV and MMP-2-1ic which seemed to have proliferated a little more slowly.
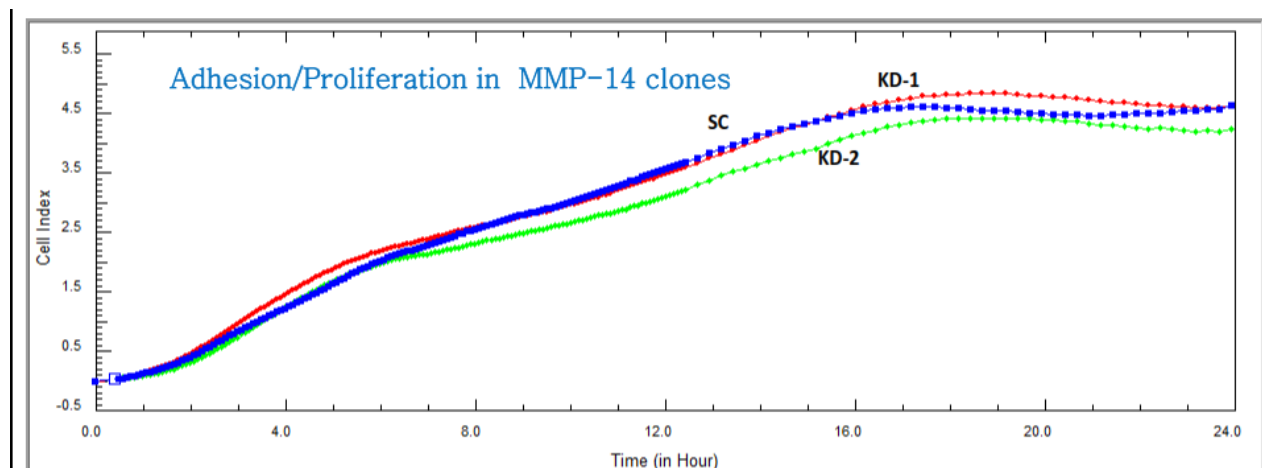
Figure 20: Adhesion and proliferation profile for MMP-14 clones
10,000 cells in 100 µl of complete growth medium (for the 8 different cell clones) were seeded in duplicates on the E-16 microplate following the equilibration of the plate wells with 50 µl medium and background reading. The samples were recorded for 24 hours.
Plate well colour code: Red: KD-1: Knockdown clone 1, Green: Knockdown clone 2 and Blue: Scramble control. The cells were monitored for 48-96 hours on the xCELLigence machine.

Figure 20, also shows a similarity in the cell adhesion/proliferation pattern across the MMP-14 clones.

The migrative capabilities of the clones was further analyzed by creating a migrative

profile in which the cells were seeded on top of the membrane in serum-free medium

and made to migrate to a more conducive environment with a chemoattractant (medium

supplemented with 10% FBS) (Figure 21).
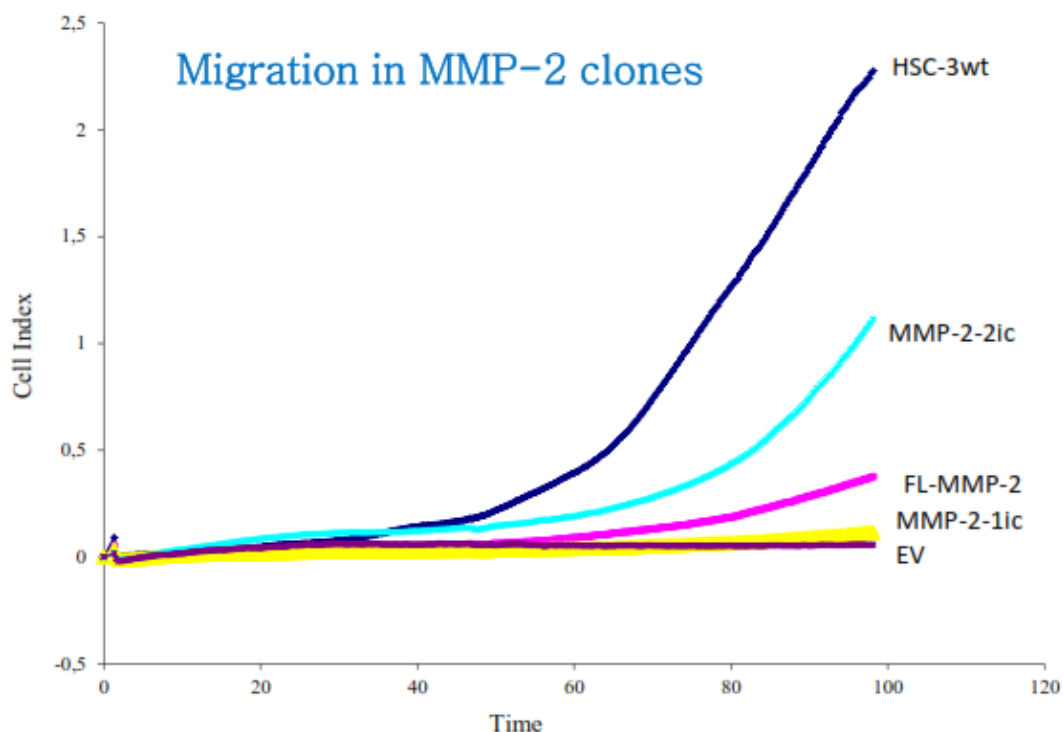
Figure 21: MMP-2 cell clones could migrate across barriers
Approximately 10,000 cells in 100 μl of SFM was seeded on the top chamber of the CIM-plate and have been made to migrate towards the bottom chamber of the plate containing medium with a chemoattractant (FBS).

Figure 21 shows varying migration patterns of the MMP-2 cell clones across the barrier

relative to the HSC-3wt. Surprisingly, FL-MMP-2 and the MMP-2-2ic migration was

slower than that of the wild type, while the MMP-2-1ic and EV showed little or no

migration.
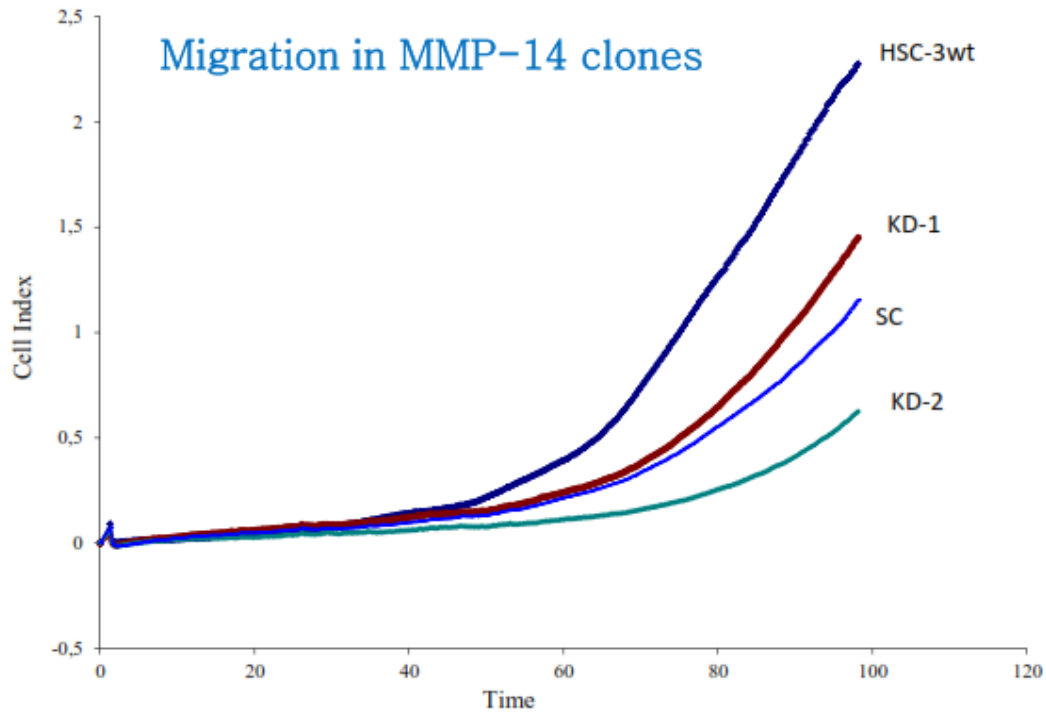
Figure 22: MMP-14 cell clones could migrate across barriers
Approximately 10,000 cells in 100 µl of SFM was seeded on the top chamber of the CIM-plate and have been made to migrate towards the bottom chamber of the plate containing medium with a chemoattractant (FBS).

Figure 22 shows that cell migration for the MMP-14 clones also follows the same pattern

of all the clones migrating much slower than the wild-type cells.
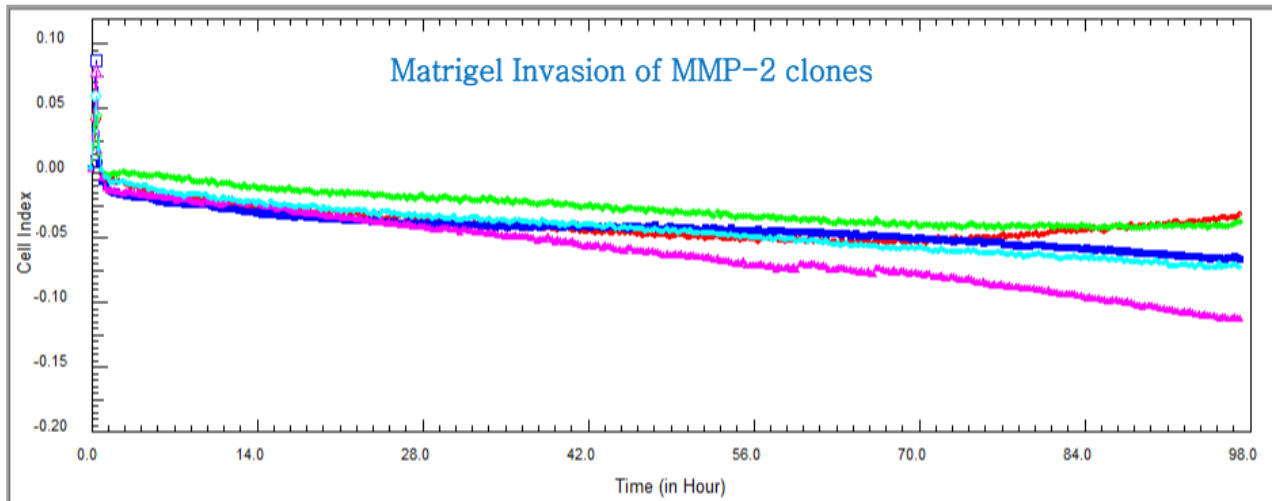
Figure 23: The MMP-2 cell clones could not invade matrigel over a 96 hour period.
xCELLigence assay for the invasion of HSC-3 MMP-2 and MMP-14 knockdown clones across matrigel was carried out by seeding 10,000 cells (in 100 μl of SFM) on the top chamber of the CIM-16 plate. The impedance is measured when the cells invade the matrigel barrier to migrate towards the chemoattractant-containing bottom chamber medium of the CIM-plate.

Figure 23 shows negligible cell indexes for all the cell clones. No invasion was observed for any of the clones after 96 hours.
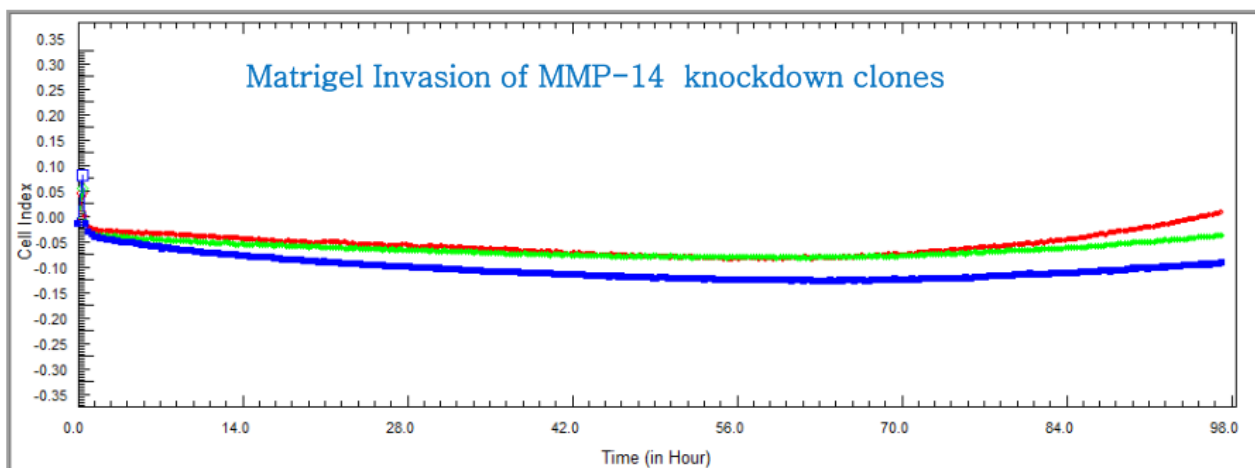
Figure 24: The MMP-14 cell clones could not invade matrigel over a 96 hour period.
xCELLigence assay for the invasion of HSC-3 MMP-2 and MMP-14 knockdown clones across matrigel was carried out by seeding 10,000 cells (in 100 μl of SFM) on the top chamber of the CIM-16 plate. The impedance is measured when the cells invade the matrigel barrier to migrate towards the chemoattractant-containing bottom chamber medium of the CIM-plate

Figure 24 shows that there was no matrigel invasion by MMP-14 cell clones in the first

72 hours of the experiment, however there was a slight in cell index afterwards.

4. DISCUSSION

Cancer can simply be defined as unregulated cell growth [104]. This definition, though very simplistic, has a much deeper meaning especially when one considers that when there is a loss of cell regulation, various pathologies can arise. Several factors can lead to cancer, but their survival is dependent on several factors which include sustaining of proliferative signalling, evasion of growth suppressors, activation of invasion and metastasis, enabling replicative immortality, induction of angiogenesis, resistance to cell death etc. [105] Cancers can arise from various types of cells, with the most common being carcinomas, which arise from epithelial cells [104]. Carcinomas account for more than 80% of cancer-related deaths and affect epithelial cell layer of the gastro intestinal tract, skin, mammary glands, pancreas, lung, liver, ovaries, gall bladder, urinary bladder etc [104]. Human Oral squamous cell carcinoma is the most common form of oral cancer. They are characterised by highly aggressive behaviour, metastasis to other sites and the prognosis is poor [55, 106].

Proteolytic enzymes have been shown to play key roles in tumor growth and invasion [77]. One of such group of enzymes are the matrix metalloproteinases [77], which have been implicated in cancer cell survival mostly due to their direct/indirect roles in normal physiological processes such as tissue remodelling in wound healing, development etc. As a result of their roles within the extracellular matrix and beyond, they are also able to enhance cell migration and invasion; thus the machineries used in normal physiological processes become arsenals in pathologies.

In this study, HSC-3 cells derived from human oral squamous cell carcinoma were used to study the roles of intracellular MMP-2 (MMP-2ic) and MMP-14 proteins by the

overexpression of MMP-2 variants and knockdown of MMP-14. This cell does not have any MMP-2 from the beginning, so for comparison, HSC-3 cells overexpressing full length MMP-2 (FL-MMP-2) was provided.

4.1 Increasing MMP-2 expression in HSC-3 cells

The expression level of MMP-2 in HSC-3 cells was first determined by Western blotting (Fig 7) in which the protein was not detectable in the sample both in the presence and absence of DTT. However, the positive control derived from conditioned fibroblast medium, showed the presence of the 72kDa MMP-2 protein and by this finding, it was assumed that the HSC-3 cells either do not endogenously express MMP-2 or they express very negligible amounts, making them good candidates for the overexpression of the protein. Expression plasmids for intracellular MMP-2 (Ex-Z5731-m02-MMP-2) and empty vector control (Ex-neg-M02) were purchased from GeneCopoeia™ USA and before use, they were amplified by transformation of *E.coli* DH5-α competent bacteria and purified. The presence of cDNA encoding intracellular MMP-2 (MMP-2ic) was verified by restriction enzyme digestion (Figure 8) and sequencing (Figure 9). Nde1 restriction enzyme digestion successfully generated two fragments each in the MMP-2ic and EV plasmids, with the band sizes corresponding to the restriction sites in the digested plasmid and corresponding to the calculated fragment sizes deduced from the plasmid map (Table 1). The three bands of the undigested (control) plasmids is most likely due to the different conformations that plasmids could exist as; circular, supercoiled or linear which influence their migration on a gel and thus, the more compact supercoiled plasmids tends to migrate faster. As expected, the plasmid containing the MMP-2ic cDNA is larger than the EV plasmid. To further confirm the presence of the MMP-2ic cDNA, DNA sequencing was performed on the purified plasmid

and BLAST search of the resulting sequences on NCBI returned about 98% complementarity between the sequenced plasmids and MMP-2transcript version 2 (encoding MMP-2ic) found in the database (Figure 9). In order to compare the intracellular version of the MMP-2 (MMP-2ic) with the full-length version (Ex-K3935-m02; FL-MMP-2), the FL-MMP-2 plasmid was also amplified by transforming the *E.coli* DH5α competent bacteria and purified.

The initial work to optimize the transfection protocol for the HSC-3 cells was performed and showed that Lipofectamine-2000 was the preferred transfection reagent and that the optimal concentration of the selection antibiotic, Geniticin (G418), should be 400μg/ml. Generation of HSC-3 single cell clones overexpressing MMP-2ic was performed and provided for further analysis by a colleague. The conditioned medium from the cells clones were analyzed for MMP-2 activity by gelatin zymography (Figure 10) and revealed that the MMP-2ic -overexpressing cells possessed gelatinolytic activity in bands with the size corresponding to MMP-2ic. No bands of that size were detected in the HSC-3-EV clones, further strengthening the assumption that the bands of approximately 42kDa corresponds to the overexpressed MMP-2ic (lanes 14 and 15). All clones also expressed pro-MMP-9 as bands of approximately 92kDa were also observed on the zymogram. Attempts to detect MMP-2ic expression by Western blotting on whole cell lysates proved futile (figure not shown). The fact that the protein was undetectable on Western blot, but detectable on the zymogram, albeit low amounts, confirms the sensitivity of zymography over western blotting, being able to detect as low as 10ρg amount of gelatinases. Again, the detection of the supposedly intracellulary overexpressed protein in conditioned serum-free medium, suggests some level of

secretion of the enzyme and also emphasizes the sensitivity of the method. Alternatively, the MMP-2ic is released into the medium from ruptured cells.

However, by virtue of this, it would have been a good idea to analyse the cell lysates by zymography in order to be able to truly ascertain if the lack of detection was due to very low amounts of the intracellular variant of the protein or its complete absence. Also, the total expression level of the MMP-2ic as shown on the zymogram, is not very strong on its own. It will not suffice to conclude at this point if the MMP-2ic protein was completely absent in the cell lysates, as some technical difficulties were encountered that also hindered the detection of the positive control. This could have been an antibiotic problem and the experiment should have been repeated a few more times. This could not be pursued due to time constraint.

The overexpression was further verified by RT-qPCR and in order to eliminate any discrepancies in the result, the purified total RNAs from the stable transfection clones which had measured concentration of ≥100 ng/µl, were sent for integrity testing (Figure 11A). The integrity testing result revealed that intact RNA (made up of the 28S and the 18S ribosomal RNA) was present in most of the samples. Two of the RNA samples were degraded to some degree and were not proceeded with in the RT-qPCR analysis. The degradation of the RNA is most likely a result of the introduction of RNases into the samples during handling. This can be avoided by ensuring to a great extent that the lab is as RNase-free as possible. Another cause of degradation could be as a result of contaminated reagents and/or water, but this is not the case due to the fact that there is no degradation across all the samples.

RT-qPCR on the intact RNAs showed variation in the relative levels of of the *MMP-2* transcripts, when compared to one of the empty vector clones (EV-7) (Figure11B). The primers used do not discriminate between between the various MMP-2 variants, however, the increased transcript levels detected by the primers is most likely due to the presence of the MMP-2ic expression vector. The second clone, MMP-2-2ic, yielded the highest expression of *MMP-2* with a fold change of 18. The variation in the MMP-2ic expression in the different clones could be attributed to the fact that plasmids taken up into a cell are able to incorporate into different positions and the more transcriptionally active the genomic region is, the more successful the expression of the incorporated vector. In order to resolve this and obtain almost identical protein expression across samples, generation of mixed clones could be employed. However, this is not a big problem in this case, since the idea was to obtain as much overexpression of the protein of interest as possible.

4.2 Reduction of MMP-14 expression in HSC-3 cells

Prior to MMP-14 knockdown, the HSC-3 cells were analysed for the inherent expression of the protein (Figure 12). Strong signals corresponding to the MMP-14 molecular weight of approximately 66kDa were detected, denoting that the cells had high amounts of the protein. Although an extra band was detected high up on the membrane, the main band was the same size as MMP-14. In order to verify the best shRNAs that would knockdown MMP-14, four different shRNA constructs were used to transiently transfect HSC-3 cells, after which equal protein concentrations were analysed by Western blotting (Figure 13A). The signal strengths of two of the constructs were relatively lower than the rest, indicating a successful knockdown in two of the clones (figure 13A). Probing for β-actin served as a loading control, in which equal bands signify that equal amount of

proteins were loaded on the gel (figure 13B). As a result of the successful knockdown, the constructs were used to stably transfect the HSC-3 cells, which again showed that MMP-14 expression was lowered using one of the shRNA constructs (Figure 14A); and with the equal actin bands, a knockdown could be said to have taken place (figure 14B). Although the main bands corresponded to the molecular weight of the 66kDa MMP-14 protein, extra bands were observed on the membrane and these can be due to protein overload, which can be corrected by reducing the amount of loaded protein or it could be due to non-specific antibody binding. The reduced band size with part of the band located lower down the membrane (Figure 14B, lane 5) for wild-type HSC-3 cell could be as a result of protein degradation. This occurs mostly due to repetitive freeze-thaw of the sample and can be avoided by using fresher samples.

The use of RT-qPCR to confirm the knockdown of MMP-14 (Figure 15), showed that both transient and stable transfections let to knockdown in clones transfected with shRNA constructs 1, 2 and 3 relative to the wildtype HSC-3 cell. It was surprising that the transiently transfected HSC-3 (KD- 3) demonstrated a stronger knockdown effect than that detected by western blotting for transient transfection (Figure 13 A). Even more surprising is the lower expression of MMP-14 in both transient and stable transfections with the scrambled control in comparison to the untransfected wild-type cells. One way to explain these is possibly sample handling, which can be improved upon in subsequent experiments. Due to the time limit, these experiments were only performed once, and have to be repeated in order to draw any conclusions.

4.3 Analysis of protein expression and subcellular localization by immunostaining and confocal microscopy

Analyses of MMP-2 expression in the various clones by immunostaining and confocal microscopy (Figure 16) revealed that the HSC-3wt cells (panel A) had some weak and diffuse fluorescent activity although they were negative for MMP-2 in Western blot analysis. This could either be as a result of autofluorescence, very low amounts of MMP-2 to which the primary antibody binds or non-specific binding of the primary/secondary antibody. These can be optimised by improving the blocking steps, using more specific antibodies and/or filtration during image acquisition. The FL-MMP-2 and MMP-2-2ic clones (panels B and D) showed some strong fluorescent activity around the cell edges, the invadopodia, while MMP-2-1ic cells (panel C) showed a more nuclear localization.

The empty vector clone (panel E) showed diffuse fluorescent patterns possibly due to autofluorescence.

The MMP-14 knockdown clones, KD-1 and KD-2 (Figure 16, panels F and G) showed some similarities in their fluorescent patterns and intensities with diffuse cytoplasmic localization and more punctuated fluorescent activity around the cell edges.

MMP-14 SC clones showed some diffuse fluorescent patterns (panel H). The stronger signals observed in the knockdown clones is rather surprising, since weaker signals compared to those of the scrambled control were expected.

This experiment though is inconclusive and must be repeated a few more times under more optimized conditions for clarity.

In situ zymography analysis on FL-MMP-2 and EV (figure 17) clones showed some gelatinolytic activity which localized to the nucleus. The gelatinolytic activity on the empty vector clone was not quite expected. The reason for this is most likely due to the presence of other gelatinases in the cells, such as MMP-9 (as can be seen in the

zymogram from figure 10). Again, the overall gelatinolytic activity in the FL-MMP-2 clone might not be solely due to MMP-2 activity and thus this finding is rather inconclusive and the experiment should be optimised, possibly by the inclusion of inhibitors in the experiments etc, and repeated before any conclusions can be drawn. The inclusion of a control with MMP-9 knockdown could be considered, although it is time consuming, it would go a long way in answering our questions. On a lighter note, the SFM control expectedly did not show any green cells.

4.4 Comparison of the MMP-2 cell clones and MMP-14 cell clones by xCELLigence: proliferation, migration and matrigel invasion

Cell migration and invasion studies by xCELLigence revealed a huge array of discrepancies. In the adhesion and proliferation assay (Figure 19), all the cell clones proliferated at almost the same rate with the exception of the empty vector clone and the MMP-2-1ic, which seemed to have fallen off from the pattern. A reason for this might be that the clones do not mimic the wild-type cell in their proliferative patterns, thus this should be ascertained separately for the individual clones. Another reason could be that less number of cells were seeded, thus the experiments should be repeated.

The migration assay (Figure 20), was not as anticipated, yielding an awry array of unexpected patterns. The observed migration pattern was HSC-3 > KD-1 > SC > MMP-2-2ic > KD-2 > FL-MMP-2 > MMP-2-1ic > EV whereas one would presume the pattern to be FL-MMP-2 > MMP-2-2ic > MMP-2-1ic > HSC-3wt > SC or EV > KD-1 or KD-2 since cells overexpressing for FL-MMP-2 were expected to migrate more than the rest and those knocked down for MMP-14 were expected to migrate far less. There is the possibility that the shRNA did not effectively knockdown the MMP-14. As can be seen from figure

16, the fold-change in MMP-14 expression in the clones with regards to that in the HSC-3 wt cell was relatively low and almost insignificant.

The invasion assay did not yield a promising result either since matrigel was not effectively invaded by any of the clones. It is possibly due to the thickness of the matrigel that could serve as a hindrance to invading cells, further diluting the matrigel could be very useful. Another fault could be with the experimental procedure, eg cell seeding errors would affect the proliferative behaviours of the cells. Trypsinization is also a factor to consider as over trypsinization of the cells would affect the cell surface receptors required for migration. Use of high passage number cells also influences their proliferation. Overall, the xCELLigence system is a very sensitive technique and care needs to be taken to optimise the experimental conditions. The results derived from the xCELLigence experiments are not conclusive since they have been done only a few number of times with non-reproducible results. However, for more certainty, the experiments have to be repeated many times over.

5. CONCLUSION

The matrix metalloproteinases -2 (MMP-2) and membrane type-1 matrix metalloproteinase (MT1-MMP) have been shown from previous studies, to interact in the promotion of tumor cell migration and invasion. The results from this study showed that HSC-3 cells were conducive for studying the MMP-2 variants and MMP-14 since they do not endogenously express MMP-2 but express high levels of MMP-14. Clones for the overexpression of the intracellular MMP-2, MMP-2ic, have been generated but more work would still need to be done for the verification of the MMP-14 knockdown. In

order to obtain more promising results, several stages have to be optimised such as: the use of alternative plasmids for MMP-2 overexpression and MMP-14 knockdown; the use of more specific antibodies for immunostaining; the inclusion of inhibitors as a control for in situ zymography; the individual determination of the proliferative patterns of cell clones in order to determine their optimal seeding densities and the optimisation of the xCELLigence experiments for reproducibility. The successful generation of MMP-14 clones, in combination with the MMP-2 clones, would provide some useful tools for further studies on MMP-2 variants and MMP-14. In the long run, further cloning would have to be done for the combined manipulation of MMP-2 and MMP-14, which will provide cells that will pose useful for studying the interaction between the two proteins. The successful establishment of these methods would go a long way in the application of MMPs as targets for cancer drugs development.

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7. Appendix

Figure 1: Alignment of the sequences of the MMP-2 vector compared with human MMP-2, transcript variant 2 (Accession number NM 001127891).

Sample 1: Forward Reaction

Score	Expect	Identities	Gaps	Strand	Frame
2015 bits(1091)	0.0()	1149/1174(98%)	15/1174(1%)	Plus/Plus	

Features:

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Query  38    CATGCAATACCTGAACACCTTCTATGGCTGCCCCAAGGAGAGCTGCAACCTGTTTGTGCT   97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314   CATGCAATACCTGAACACCTTCTATGGCTGCCCCAAGGAGAGCTGCAACCTGTTTGTGCT   373

Query  98    GAAGGACACACTAAAGAAGATGCAGAAGTTCTTTGGACTGCCCCAGACAGGTGATCTTGA   157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374   GAAGGACACACTAAAGAAGATGCAGAAGTTCTTTGGACTGCCCCAGACAGGTGATCTTGA   433

Query  158   CCAGAATACCATCGAGACCATGCGGAAGCCACGCTGCGGCAACCCAGATGTGGCCAACTA   217
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434   CCAGAATACCATCGAGACCATGCGGAAGCCACGCTGCGGCAACCCAGATGTGGCCAACTA   493

Query  218   CAACTTCTTCCCTCGCAAGCCCAAGTGGGACAAGAACCAGATCACATACAGGATCATTGG   277
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494   CAACTTCTTCCCTCGCAAGCCCAAGTGGGACAAGAACCAGATCACATACAGGATCATTGG   553

Query  278   CTACACACCTGATCTGGACCCAGAGACAGTGGATGATGCCTTTGCTCGTGCCTTCCAAGT   337
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  554   CTACACACCTGATCTGGACCCAGAGACAGTGGATGATGCCTTTGCTCGTGCCTTCCAAGT   613

Query  338   CTGGAGCGATGTGACCCCACTGCGGTTTTCTCGAATCCATGATGGAGAGGCAGACATCAT   397
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  614   CTGGAGCGATGTGACCCCACTGCGGTTTTCTCGAATCCATGATGGAGAGGCAGACATCAT   673

Query  398   GATCAACTTTGGCCGCTGGGAGCATGGCGATGGATACCCCTTTGACGGTAAGGACGGACT   457
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  674   GATCAACTTTGGCCGCTGGGAGCATGGCGATGGATACCCCTTTGACGGTAAGGACGGACT   733

Query  458   CCTGGCTCATGCCTTCGCCCCAGGCACTGGTGTTGGGGGAGACTCCCATTTTGATGACGA   517
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734   CCTGGCTCATGCCTTCGCCCCAGGCACTGGTGTTGGGGGAGACTCCCATTTTGATGACGA   793

Query  518   TGAGCTATGGACCTTGGGAGAAGGCCAAGTGGTCCGTGTGAAGTATGGCAACGCCGATGG   577
             |||||||||||||||||||||||||||||||||||||||||||||||  |||||||||||
Sbjct  794   TGAGCTATGGACCTTGGGAGAAGGCCAAGTGGTCCGTGTGAAGTATGGGAACGCCGATGG   853

Query  578   GGAGTACTGCAAGTTCCCCTTCTTGTTCAATGGCAAGGAGTACAACAGCTGCACTGATAC   637
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  854   GGAGTACTGCAAGTTCCCCTTCTTGTTCAATGGCAAGGAGTACAACAGCTGCACTGATAC   913

Query  638   CGGCCGCAGCGATGGCTTCCTCTGGTGCTCCACCACCTACAACTTTGAGAAGGATGGCAA   697
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  914   CGGCCGCAGCGATGGCTTCCTCTGGTGCTCCACCACCTACAACTTTGAGAAGGATGGCAA   973

Query  698   GTACGGCTTCTGTCCCCATGAAGCCCTGTTCACCATGGGCGGCAACGCTGAAGGACAGCC   757
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  974   GTACGGCTTCTGTCCCCATGAAGCCCTGTTCACCATGGGCGGCAACGCTGAAGGACAGCC   1033

Query  758   CTGCAAGTTTCCATTCCGCTTCCAGGGCACATCCTATGACAGCTGCACCACTGAGGGCCG   817
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1034  CTGCAAGTTTCCATTCCGCTTCCAGGGCACATCCTATGACAGCTGCACCACTGAGGGCCG   1093

Query  818   CACGGATGGCTACCGCTGGTGCGGCACCACTGAGGACTACGACCGCGACAAGAAGTATGG   877
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1094  CACGGATGGCTACCGCTGGTGCGGCACCACTGAGGACTACGACCGCGACAAGAAGTATGG   1153

Query  878   CTTCTGCCCTGAGACCGCCATGTCCACTGTTGGTGGGAACTCAGAA-GTGCCCCCTGTGT   936
             |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
Sbjct  1154  CTTCTGCCCTGAGACCGCCATGTCCACTGTTGGTGGGAACTCAGAAGGTGCCCCCTGTGT   1213

Query  937   CTTCCCCTTCACTTTCCTGGGCAACAAATATGAGAGCTGCACCAGCGCCGGCCGCAGTGA   996
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1214  CTTCCCCTTCACTTTCCTGGGCAACAAATATGAGAGCTGCACCAGCGCCGGCCGCAGTGA   1273

Query  997   CGGAAAGATGTGGTGTGCGACCACAGCCAACTACGATGACGACCGCAAGTGGGGGCTTCT   1056
             |||||||||||||||||||||||||||||||||||||||| ||||||||||| |||||||
Sbjct  1274  CGGAAAGATGTGGTGTGCGACCACAGCCAACTACGATGATGACCGCAAGT-GGGGCTTCT   1332

Query  1057  GCCCTGACCAAAGGTACAGCCTGTTC-TCGT-GCAGCCCACGAGTTTGGGCACGGCATGG   1114
             |||||||||| |||||||||||||||| |||| |||||||||||||||||| |||| |||||
Sbjct  1333  GCCCTGACCAAGGGTACAGCCTGTTCCTCGTGGCAGCCCACGAGTTTGGCCACGCCATGG   1392

Query  1115  GGCT-GAGCACTCCCAGGACC-TGGGGACCCTGATG-CACCCATT-ACACTTAC-C-AGG   1168
             |||| |||||||||||| |||| |||||| |||||||| ||||||||| |||| ||| | | |
Sbjct  1393  GGCTGGAGCACTCCCAAGACCCTGGGG-CCCTGATGGCACCCATTTACACCTACACCAAG   1451

Query  1169  A-CTCCGGTCTGTCC-AGGATGAACACTCAAGGG    1200
             | || | |||||||| ||||||| || |||||||
Sbjct  1452  AACTTCCGTCTGTCCCAGGATGA-CA-TCAAGGG    1483
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Sample 1: Reverse reaction

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Query  58    AGCT-AGCAGCCTAGCCAGTCGGATTTGATGCTTCCAAACTTCACGCTCTTCAGACTTTG   116
             |||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2150  AGCTCAGCAGCCTAGCCAGTCGGATTTGATGCTTCCAAACTTCACGCTCTTCAGACTTTG   2091

Query  117   GTTCTCCAGCTTCAGGTAATAGGCACCCTTGAAGAAGTAGCTGTGACCGCCGCCCTGCAG   176
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2090  GTTCTCCAGCTTCAGGTAATAGGCACCCTTGAAGAAGTAGCTGTGACCGCCGCCCTGCAG   2031

Query  177   GTCCACGACGGCATCCAGGTTATCGGGGATGGCATTCCAGGCATCTGCGATGAGCTTGGG   236
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2030  GTCCACGACGGCATCCAGGTTATCGGGGATGGCATTCCAGGCATCTGCGATGAGCTTGGG   1971

Query  237   AAAGCCAGGATCCATTTTCTTCTTCACCTCATTGTATCTCCAGAATTTGTCTCCAGCAAA   296
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1970  GAAGCCAGGATCCATTTTCTTCTTCACCTCATTGTATCTCCAGAATTTGTCTCCAGCAAA   1911

Query  297   GATGTATGTCTTCTTGTTTTTTGCTCCAGTTAAAGGCGGCATCCACTCGCTGGACATCAGG   356
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1910  GATGTATGTCTTCTTGTTTTTTGCTCCAGTTAAAGGCGGCATCCACTCGCTGGACATCAGG   1851

Query  357   GGGCAGTCCCAGGCTGGTCAGTGGCTTGGGGTACCCTCGCTCCAGGGTGCTGGCTGAGTA   416
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1850  GGGCAGTCCCAGGCTGGTCAGTGGCTTGGGGTACCCTCGCTCCAGGGTGCTGGCTGAGTA   1791

Query  417   GATCCAGTATTCATTCCCTGCAAAGAACACAGCCTTCTCCTCCTGTGGGGCCTCGTATAC   476
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1790  GATCCAGTATTCATTCCCTGCAAAGAACACAGCCTTCTCCTCCTGTGGGGCCTCGTATAC   1731

Query  477   CGCATCAATCTTTTCCGGGAGCTCAGGCCAGAATGTGGCCACCAGCAGGGGCCCCATGGG   536
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1730  CGCATCAATCTTTTCCGGGAGCTCAGGCCAGAATGTGGCCACCAGCAGGGGCCCCATGGG   1671
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Query  537   CTTGTCACGTGGCGTCACAGTCCGCCAAATGAACCGGTCCTTGAAGAAGAAGATCTCACC   596
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1670  CTTGTCACGTGGCGTCACAGTCCGCCAAATGAACCGGTCCTTGAAGAAGAAGATCTCACC   1611

Query  597   ACGGATCTGAGCGATGCCATCAAATACAATGTCCTGTTTGCAGATCTCAGGAGTGACAGG   656
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1610  ACGGATCTGAGCGATGCCATCAAATACAATGTCCTGTTTGCAGATCTCAGGAGTGACAGG   1551

Query  657   GCCCAGTGTGGGGGTGGGGCCGGTGCCAAGGTCAATGTCAGGAGAGGCCCCATAGAGCTC   716
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1550  GCCCAGCGTGGGGGTGGGGCCGGTGCCAAGGTCAATGTCAGGAGAGGCCCCATAGAGCTC   1491

Query  717   CTGAATGCCCTTGATGTCATCCTGGGACAGACGGAAGTTCTTGGTGTAGGTGTAAATGGG   776
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1490  CTGAATGCCCTTGATGTCATCCTGGGACAGACGGAAGTTCTTGGTGTAGGTGTAAATGGG   1431

Query  777   TGCCATCAGGGCCCCAGGGTCTTGGGAGTGCTCCAGCCCCATGGCGTGGCCAAACTCGTG   836
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1430  TGCCATCAGGGCCCCAGGGTCTTGGGAGTGCTCCAGCCCCATGGCGTGGCCAAACTCGTG   1371

Query  837   GGCTGCCACGAGGAACAGGCTGTACCCTTGGTCAGGGCAGAAGCCCCACTTGCGGTCGTC   896
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1370  GGCTGCCACGAGGAACAGGCTGTACCCTTGGTCAGGGCAGAAGCCCCACTTGCGGTCATC   1311

Query  897   ATCGTAGTTGGCTGTGGTCGCACACCACATCTTTCCGTCACTGCGGCCGGCGCTGGTGCA   956
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1310  ATCGTAGTTGGCTGTGGTCGCACACCACATCTTTCCGTCACTGCGGCCGGCGCTGGTGCA   1251

Query  957   GCTCTCATATTTGTTGCCCAGGAAAGTGAATGGGAAGACACAGGGG-CACCTTTCTGAGT   1015
             |||||||||||||||||||||||||||| |||||||||||||||| |||| |||||||||
Sbjct  1250  GCTCTCATATTTGTTGCCCAGGAAAGTGAAGGGGAAGACACAGGGGGCACC-TTCTGAGT   1192

Query  1016  TCCCACCAACAGTGGACATGGCGGTCTCAGG-CAG-AGCCATACTTCTTGTCGCGGTCGT   1073
             ||||||||||||||||||||||||||||||| ||| ||||||||||||||||||||||||
Sbjct  1191  TCCCACCAACAGTGGACATGGCGGTCTCAGGGCAGAAGCCATACTTCTTGTCGCGGTCGT   1132

Query  1074  AGTC-TCAGT-GTGCCGCACCAGCG-TAGCCATCCGTGCG-CCCTCAAT-GTGCAGCTGT   1128
             |||| ||||| |||||||||||||| |||||||||||||| |||||| | ||||||||||
Sbjct  1131  AGTCCTCAGTGGTGCCGCACCAGCGGTAGCCATCCGTGCGGCCCTCAGTGGTGCAGCTGT   1072

Query  1129  CATAGGAATGTGCC-TGGA-GCG-A-TGGAAAACGTGCAG--CTGTCCT-CA-CGATG-C   1179
             |||||||| |||||| |||| ||| | ||||| | ||||| ||||||| || || |||| |
Sbjct  1071  CATAGGA-TGTGCCCTGGAAGCGGAATGGAAA-CTTGCAGGGCTGTCCTTCAGCGTTGCC   1014

Query  1180  GCCAATGGTGAACAG   1194
             ||| |||||||||||
Sbjct  1013  GCCCATGGTGAACAG   999
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Sample 2:
Forward reaction

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Query  37    CATGCAATACCTGAACACCTTCTATGGCTGCCCCAAGGAGAGCTGCAACCTGTTTGTGCT   96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314   CATGCAATACCTGAACACCTTCTATGGCTGCCCCAAGGAGAGCTGCAACCTGTTTGTGCT   373

Query  97    GAAGGACACACTAAAGAAGATGCAGAAGTTCTTTGGACTGCCCCAGACAGGTGATCTTGA   156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374   GAAGGACACACTAAAGAAGATGCAGAAGTTCTTTGGACTGCCCCAGACAGGTGATCTTGA   433

Query  157   CCAGAATACCATCGAGACCATGCGGAAGCCACGCTGCGGCAACCCAGATGTGGCCAACTA   216
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434   CCAGAATACCATCGAGACCATGCGGAAGCCACGCTGCGGCAACCCAGATGTGGCCAACTA   493

Query  217   CAACTTCTTCCCTCGCAAGCCCAAGTGGGACAAGAACCAGATCACATACAGGATCATTGG   276
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494   CAACTTCTTCCCTCGCAAGCCCAAGTGGGACAAGAACCAGATCACATACAGGATCATTGG   553
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Query   277    CTACACACCTGATCTGGACCCAGAGACAGTGGATGATGCCTTTGCTCGTGCCTTCCAAGT    336
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   554    CTACACACCTGATCTGGACCCAGAGACAGTGGATGATGCCTTTGCTCGTGCCTTCCAAGT    613

Query   337    CTGGAGCGATGTGACCCCACTGCGGTTTTCTCGAATCCATGATGGAGAGGCAGACATCAT    396
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   614    CTGGAGCGATGTGACCCCACTGCGGTTTTCTCGAATCCATGATGGAGAGGCAGACATCAT    673

Query   397    GATCAACTTTGGCCGCTGGGAGCATGGCGATGGATACCCCTTTGACGGTAAGGACGGACT    456
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   674    GATCAACTTTGGCCGCTGGGAGCATGGCGATGGATACCCCTTTGACGGTAAGGACGGACT    733

Query   457    CCTGGCTCATGCCTTCGCCCCAGGCACTGGTGTTGGGGGAGACTCCCATTTTGATGACGA    516
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   734    CCTGGCTCATGCCTTCGCCCCAGGCACTGGTGTTGGGGGAGACTCCCATTTTGATGACGA    793

Query   517    TGAGCTATGGACCTTGGGAGAAGGCCAAGTGGTCCGTGTGAAGTATGGCAACGCCGATGG    576
               |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct   794    TGAGCTATGGACCTTGGGAGAAGGCCAAGTGGTCCGTGTGAAGTATGGGAACGCCGATGG    853

Query   577    GGAGTACTGCAAGTTCCCCTTCTTGTTCAATGGCAAGGAGTACAACAGCTGCACTGATAC    636
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   854    GGAGTACTGCAAGTTCCCCTTCTTGTTCAATGGCAAGGAGTACAACAGCTGCACTGATAC    913

Query   637    CGGCCGCAGCGATGGCTTCCTCTGGTGCTCCACCACCTACAACTTTGAGAAGGATGGCAA    696
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   914    CGGCCGCAGCGATGGCTTCCTCTGGTGCTCCACCACCTACAACTTTGAGAAGGATGGCAA    973

Query   697    GTACGGCTTCTGTCCCCATGAAGCCCTGTTCACCATGGGCGGCAACGCTGAAGGACAGCC    756
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   974    GTACGGCTTCTGTCCCCATGAAGCCCTGTTCACCATGGGCGGCAACGCTGAAGGACAGCC    1033

Query   757    CTGCAAGTTTCCATTCCGCTTCCAGGGCACATCCTATGACAGCTGCACCACTGAGGGCCG    816
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1034   CTGCAAGTTTCCATTCCGCTTCCAGGGCACATCCTATGACAGCTGCACCACTGAGGGCCG    1093

Query   817    CACGGATGGCTACCGCTGGTGCGGCACCACTGAGGACTACGACCGCGACAAGAAGTATGG    876
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1094   CACGGATGGCTACCGCTGGTGCGGCACCACTGAGGACTACGACCGCGACAAGAAGTATGG    1153

Query   877    CTTCTGCCCTGAGACCGCCATGTCCACTGTTGGTGGGAACTCAGAA-GTGCCCCCTGTGT    935
               |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
Sbjct   1154   CTTCTGCCCTGAGACCGCCATGTCCACTGTTGGTGGGAACTCAGAAGGTGCCCCCTGTGT    1213

Query   936    CTTCCCCTTCACTTTCCTGGGC-ACAAATATGAGAGCTGCACCAGCGCCG-CCGCAGTGA    993
               ||||||||||||||||||||||| ||||||||||||||||||||||||||| |||||||||
Sbjct   1214   CTTCCCCTTCACTTTCCTGGGCAACAAATATGAGAGCTGCACCAGCGCCGGCCGCAGTGA    1273

Query   994    CG-AAAGATGT-GTGTGCGACCACAGCC-ACTACGATGACGACCGCAAGTGGGGGCTTCT    1050
               || ||||||||| |||||||||||||||| |||||||||| ||||||||| ||||||||||
Sbjct   1274   CGGAAAGATGTGGTGTGCGACCACAGCCAACTACGATGATGACCGCAAGT-GGGGCTTCT    1332

Query   1051   GCCCTGACCAAGG-TACAGCCTGT-C-TCGT-GCAGCCCACGAG-TTGTCCACGCCATGG    1105
               |||||||||||| ||||||||| | |||| ||||||||||| ||| ||||||||||
Sbjct   1333   GCCCTGACCAAGGGTACAGCCTGTTCCTCGTGGCAGCCCACGAGTTTGGCCACGCCATGG    1392

Query   1106   GGCTGGAGCACTCCCA-GAC--TGGGGC--TGATG-CACCC    1140
               |||||||||||||||| ||| |||||| ||||| |||||
Sbjct   1393   GGCTGGAGCACTCCCAAGACCCTGGGGCCCTGATGGCACCC    1433
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Reverse reaction
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Query   57     AGCT-AGCAGCCTAGCCAGTCGGATTTGATGCTTCCAAACTTCACGCTCTTCAGACTTTG    115
               |||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2150   AGCTCAGCAGCCTAGCCAGTCGGATTTGATGCTTCCAAACTTCACGCTCTTCAGACTTTG    2091

Query   116    GTTCTCCAGCTTCAGGTAATAGGCACCCTTGAAGAAGTAGCTGTGACCGCCGCCCTGCAG    175
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                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2090  GTTCTCCAGCTTCAGGTAATAGGCACCCTTGAAGAAGTAGCTGTGACCGCCGCCCTGCAG  2031

Query   176  GTCCACGACGGCATCCAGGTTATCGGGGATGGCATTCCAGGCATCTGCGATGAGCTTGGG   235
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2030  GTCCACGACGGCATCCAGGTTATCGGGGATGGCATTCCAGGCATCTGCGATGAGCTTGGG  1971

Query   236  AAAGCCAGGATCCATTTTCTTCTTCACCTCATTGTATCTCCAGAATTTGTCTCCAGCAAA   295
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1970  GAAGCCAGGATCCATTTTCTTCTTCACCTCATTGTATCTCCAGAATTTGTCTCCAGCAAA  1911

Query   296  GATGTATGTCTTCTTGTTTTTGCTCCAGTTAAAGGCGGCATCCACTCGCTGGACATCAGG   355
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1910  GATGTATGTCTTCTTGTTTTTGCTCCAGTTAAAGGCGGCATCCACTCGCTGGACATCAGG  1851

Query   356  GGGCAGTCCCAGGCTGGTCAGTGGCTTGGGGTACCCTCGCTCCAGGGTGCTGGCTGAGTA   415
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1850  GGGCAGTCCCAGGCTGGTCAGTGGCTTGGGGTACCCTCGCTCCAGGGTGCTGGCTGAGTA  1791

Query   416  GATCCAGTATTCATTCCCTGCAAAGAACACAGCCTTCTCCTCCTGTGGGGCCTCGTATAC   475
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1790  GATCCAGTATTCATTCCCTGCAAAGAACACAGCCTTCTCCTCCTGTGGGGCCTCGTATAC  1731

Query   476  CGCATCAATCTTTTCCGGGAGCTCAGGCCAGAATGTGGCCACCAGCAGGGGCCCCATGGG   535
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1730  CGCATCAATCTTTTCCGGGAGCTCAGGCCAGAATGTGGCCACCAGCAGGGGCCCCATGGG  1671

Query   536  CTTGTCACGTGGCGTCACAGTCCGCCAAATGAACCGGTCCTTGAAGAAGAAGATCTCACC   595
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1670  CTTGTCACGTGGCGTCACAGTCCGCCAAATGAACCGGTCCTTGAAGAAGAAGATCTCACC  1611

Query   596  ACGGATCTGAGCGATGCCATCAA---CAATGTCCTGTTTGCAGATCTCAGGAGTGACAGG   652
                    ||||||||||||||||||||||||   |||||||||||||||||||||||||||||||||
Sbjct  1610  ACGGATCTGAGCGATGCCATCAAATACAATGTCCTGTTTGCAGATCTCAGGAGTGACAGG  1551

Query   653  GCCCAGTGTGGGGGTGGGGCCGGTGCCAAGGTCAATGTCAGGAGAGGCCCCATAGAGCTC   712
                    ||||||   |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1550  GCCCAGCGTGGGGGTGGGGCCGGTGCCAAGGTCAATGTCAGGAGAGGCCCCATAGAGCTC  1491

Query   713  CTGAATGCCCTTGATGTCATCCTGGGACAGACGGAAGTTCTTGGTGTAGGTGTAAATGGG   772
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1490  CTGAATGCCCTTGATGTCATCCTGGGACAGACGGAAGTTCTTGGTGTAGGTGTAAATGGG  1431

Query   773  TGCCATCAGGGCCCCAGGGTCTTGGGAGTGCTCCAGCCCCATGGCGTGGCCAAACTCGTG   832
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1430  TGCCATCAGGGCCCCAGGGTCTTGGGAGTGCTCCAGCCCCATGGCGTGGCCAAACTCGTG  1371

Query   833  GGCTGCCACGAGGAACAGGCTGTACCCTTGGTCAGGGCAGAAGCCCCACTTGCGGTCGTC   892
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1370  GGCTGCCACGAGGAACAGGCTGTACCCTTGGTCAGGGCAGAAGCCCCACTTGCGGTCATC  1311

Query   893  ATCGTAGTTGGCTGTGGTCGCACACCACATCTTTCCGTCACTGCGGCCGGCGCTGGTGCA   952
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1310  ATCGTAGTTGGCTGTGGTCGCACACCACATCTTTCCGTCACTGCGGCCGGCGCTGGTGCA  1251

Query   953  GCTCTCATATTTGTTGCCCAGGAAAGTGAAAGGGGAAGACACAGGGG-CACCTTCTGAGT  1011
                    |||||||||||||||||||||||||||||||||| ||||||||||||||||| ||||||||
Sbjct  1250  GCTCTCATATTTGTTGCCCAGGAAAGTGAA-GGGGAAGACACAGGGGGCACCTTCTGAGT  1192

Query  1012  TCCCACCAACAGTGGACATGGCGGTCTCAG--CAGA-GCCATACTTTCTTGTCGCGGTCG  1068
                    ||||||||||||||||||||||||||||||  |||| ||||||| |||||||||||||||
Sbjct  1191  TCCCACCAACAGTGGACATGGCGGTCTCAGGGCAGAAGCCATAC-TTCTTGTCGCGGTCG  1133

Query  1069  TAGTC-TCAGTGGTGCCGCACCAGCGGTTAGC-AT-CGTGCGTCCCTCAGTGA-GCAGCC  1124
                    ||||| ||||||||||||||||||||||| ||| || ||||||| ||||||||| |||||
Sbjct  1132  TAGTCCTCAGTGGTGCCGCACCAGCGGT-AGCCATCCGTGCGGCCCTCAGTGGTGCAGC-  1075

Query  1125  TTGTCATAGAATGTGCC-TG-A-GCGGGATTGGAAACCTGCAGC-CTGTTCCTTTCAAGC  1180
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Sbjct  1074   -TGTCATAGGATGTGCCCTGGAAGCGGAA-TGGAAACTTGCAGGGCTGT-CCTT-CA-GC   1020

Query  1181   GATTGACCGGCCC   1193
              | ||| ||| |||
Sbjct  1019   G-TTG-CCG-CCC   1010
```